Declining Male Fertility: Investigations into an Environmental Aetiology using a Canine Model.

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Abstract:

Contemporary reports showing negative temporal trends in human sperm quality have provided a weight of evidence, in spite of recognised methodological weaknesses of the early research, strongly indicative of declining male fertility over the past six decades. Other aspects of human male reproductive development, such as hypospadias, cryptorchidism and testis cancer are also showing negative trends in incidence and geographic variation in prevalence. Together these negative changes in parameters of fertility, which have comorbidity, are termed Testicular Dysgenesis Syndrome (TDS). The relative short time period of the trend data and the spatial heterogeneity of these factors suggest an environmental aetiology. Two neighboring and ethno-linguistic similar countries, Denmark and Finland, are used as evidence of this as Denmark has significantly decreased sperm quality as well as an increased incidence of other aspects of TDS when compared to neighboring Finland. Additionally, exposure to persistent environmental chemical pollutants has been associated with decreased sperm quality and increases in other components of TDS. The suggested underlying cause of TDS is perturbed foetal/embryonal programming and reproductive development. In spite of overall poor epidemiological information and a paucity of empirical research data from companion animals there are suggestions that environmental pollutants are affecting the health of cats and dogs. Indeed, the dog may be a relevant research model as it shares our environment and ill health associated with it, such as human TDS. Based on the evidence of environmental pollutants affecting human and animal reproductive development and fertility, this PhD project was developed in order to determine if man's closest companion, and the animal which wholly shares our environment, demonstrates any parallel reproductive impairment. Specifically, this thesis aims to investigate negative temporal trends related to canine fertility, determine the presence of and geographic variation in tissue concentrations of exemplar environmental pollutants and examine testes tissue and sperm cells, via culture and histology, for association between chemical concentrations and quantitative assessments.

Canine testes were collected from three countries; UK (n = 58), Finland (Helsinki region) (n = 20), Denmark (Copenhagen region) (n = 10). These samples were tested for concentrations of three representative chemical groups: PCBs, PBDEs, DEHP. Samples of commercial dog food (n = 28), and a range of canine body fluid was also analysed for these same chemical groups. A retrospective analysis of database information from a population of service dogs in the UK was used to determine temporal trends in canine sperm quality and cryptorchidism over a 26 year period. The concentrations of the three chemical pollutant types were additionally analysed against land use with a Geographic Information System (GIS) and two CORINE land use categories: Artificial and Agricultural. These tissues were assessed histologically using histomorphometric scores, CYP11A1 area stained. Canine testes tissue and sperm were cultured with PCB153 and DEHP to determine direct effects of short term exposures to environmental pollutants by assessing testosterone production (testes explant culture), motility (CASA), sperm viability (hypo osmotic swelling test, chromatin integrity, live:dead ratio). Database analysis of service dog fertility reported two components of TDS: negative temporal trends in sperm quality and a concurrent increased incidence of cryptorchidism. Samples of canine testes tissue contained measurable concentrations of three exemplar pollutants (PCBs, PBDEs, DEHP). These pollutants were also largely present in commercial dog food and some were also present in prostatic fluid, full ejaculate and bitches milk. The profile of EDs and concentrations of specific chemicals showed significant variation across three regions of the UK (Southeast, East Midlands, and West Midlands). Notably, concentrations of legacy industrial chemicals (PCBs, PBDEs) were highest in the

West Midlands and phthalate (DEHP) was highest in the southeast. The concentrations of DEHP was significantly higher in Artificial land categories compared to tissue collected in Agricultural land use areas ($P<0.05$). Canine testis samples collected from three international regions contained concentrations of PCBs and DEHP which were significantly higher in the UK ($P<0.05$) and PBDEs which were significantly higher in Finland ($P<0.05$). The histomorphometric assessment of testes showed significant variations across the three countries. The quantification of immunohistochemical testis area stained for CYP11A1 from the three countries showed a significant variation ($P=0.0001$), and tissue from Denmark showed a significant association between area stained and concentrations of DEHP ($P<0.05$). Tissue exposed, via testes explant culture, to PCB153 and DEHP at two concentrations showed no significant blunting of LH stimulated testosterone secretion. Sperm exposed, via culturing with two environmentally relevant concentrations of PCB153 and DEHP, exhibited significantly increased ($P<0.05$) and decreased ($P<0.05$) measures of motility with these chemicals respectively. Additionally sperm viability, measured using the hypo osmotic swelling assay, was significantly reduced in the presence of PCB153 ($P<0.05$) and a significant increased when cultured with DEHP ($P<0.01$). Both chemicals significantly increased sperm DNA fragmentation. Additionally, short term exposure to both chemicals significantly reduced the Live:Dead ratio of sperm ($P<0.05$).

Robust, determinative evidence of negative trends in canine sperm quality and incidence of cryptorchidism indicate that canine and human male fertility are decreasing in parallel. An environmental aetiology is supported by the presence of environmental chemicals in canine diet, testes tissue, prostatic fluid and full canine ejaculate. Geographic differences (National and International) in testis chemical profiles and concentrations may equate with reported regional differences in human TDS. In support of this hypothesis, specific canine testis chemical concentrations were associated with histomorphometric scores from the same cohort of samples. Direct effects of PCB153 and DEHP on sperm motility and viability may be indicative of an alternative acute negative effect on male fertility. These data suggest that the dog, while living in a shared environment with humans, exhibits some parameters of TDS and that canine, as well human, male fertility is declining due to exposure to environmental chemicals.

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AMH - Anti-Mullërian Hormone.

AGD – Anogenital distance.

AR – Androgen receptor.

B/Bo% - Percent Bound

BPH - Benign prostate hyperplasia.

BPA - Biphenyl A.

CAS - Chemical Abstracts Service.

CEMP - Co-ordinated Environmental Monitoring Programme.

CIS - carcinoma *in situ*.

CLC – CORINE land cover.

CORINE - Coordination of information on the environment.

CS - Human carnegie stages.

CV = Coefficient of variation.

CYP11a1 - Cytochrome P450 family 11 subfamily A member 1.

DB cAMP - Dibutyryl cyclic- Adenosine monophosphate.

DEHP – diethylhexyl phthalate; di(2-ethylhexyl)phthalate.

DDT - dichlorodiphenyl trichloroethane.

DHT – Dihydrotestosterone.

DMEM – Dulbeco's modified eagle's serum.

DPC - Days post coitum.

E2 - Oestrodial

EC – European Commission.

ED - endocrine disruptor.

ED - endocrine disrupting chemical; endocrine disrupting compound.

EPA – Environmental Protection Agency.

ETS - Environmental tobacco smoke.

EU – European Union.

FSH - Follicle Stimulating hormone.

FO – Frequency of occurrence.

GC – Gas chromatography.

GEBCO - General Bathymetric Chart of the Oceans.

GnRH - Gonadotropin releasing hormone.

GIS – geographic information system.

GNIS - germ cell neoplasia *in situ* cells

HE - Haematoxylin and eosin.

HPG _ Hypothalamic pituitary gonadal axis.

ICES - International Council for the Exploration of the Sea.

IMC – Immunohistochemistry.

INSL3 - Insulin-like 3

ISO - International Organization for Standardization.

LD – Limit of detection.

LD_{50} – Lethal dose, 50%

LH - Luteinizing hormone.

LOAELs – Lowest Observable Effect Concentrations.

NGA – National Geospatial-Intelligence Agency.

NMDRC – Non monotonic dose response curve.

NOAA - National Oceanic and Atmospheric Administration.

NOAEL - Non Observed Adverse Effect Level.

OD -net optical density

PBDE -Polybrominated diphenyl ethers.

PBS - Phosphate-buffered saline buffer.

PCBs - polychlorinated biphenyls.

PMN – Percentage normal motility.

PNS – Percentage normal sperm.

PTM - Peritubular myoid cells.

PVC - Polyvinyl chloride.

QT –Quality control.

QM – Quality management.

REACH - Registration, Evaluation, Authorisation and Restriction of Chemicals.

RO – Reverse osmosis.

SARS - Severe acute respiratory syndrome.

SCD - Sperm Chromatin Dispersion test.

SCSA - Sperm Chromatic Structural Assay.

SRY – Sex Determining Region Y.

SIO - Scripps Institution of Oceanography.

SVHC - substance of very high concern.

TDF – Testes Determining Factor.

TDS – testicular dysgenesis syndrome.

TSO – Total sperm output.

Tunel - Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labelling.

UNEP – United Nations Environment Programme

VCCEP - Voluntary Children's Chemical Evaluation Program.

WHO – World Health Organisation.

YO – years old.

Chapter 1 INTRODUCTION AND LITERATURE REVIEW

1.1 Background and Literature

A number of epidemiological studies indicate that there has been, in the last 60 years, a significant change in parameters related to human male fertility (Toppari et al., 1996). Indicators of reproductive health such as cryptorchidism, testis cancer (specifically teratocarcinoma) and hypospadias have shown adverse trends over a similar time period. Fertility factors and measures of reproductive health have an interrelationship (Katrine Bay, Asklund, Skakkebaek, & Andersson, 2006) and are collectively termed Testicular Dysgenesis Syndrome (TDS). All components of TDS (arguably with the exception of sperm quality) are indicative of perturbed prenatal development resulting in adult pathology. This syndrome has been attributed to anthropogenic chemicals or compounds found in the environment, some of which alter normal endocrine function.

The time period of the reported decline in factors related to fertility and adverse trends in male reproductive development suggest these changes have an environmental rather than genetic aetiology. This theory is supported by geographic variations in measures of fertility and other aspects of TDS. The example of Denmark and Finland, two Scandinavian countries, with differences reported in sperm quality, has been put forward as evidence of this environmental phenomenon.

The group of environmental pollutants labelled as endocrine disruptors are suggested to perturb reproductive development and function and these compounds, along with other chemical pollutants, are credited with causing or contributing to the reported decline in male fertility. The variation in environmental ubiquity of these chemical pollutants may be reflected in the male fertility trends. These trends and their causes have significant and troubling implications for human health, as discussed in the next section.

1.2 TEMPORAL TRENDS IN HUMAN MALE SEMEN QUALITY

A publication by Carlsen et al. (1992) 'Evidence for decreasing quality of semen for the last 50 years.' initiated a debate on the reality of negative temporal trends in human semen quality. This meta-analysis amalgamated results from 61 studies across a number of countries over a period from 1938 – 1991. It included 14,946 men and showed a significant decline in two parameters related to semen quality. Figure 1.1 shows the results for one of these parameters: change in mean sperm density from 1940 – 1990 ($113 \times 10^6 - 66 \times 10^6$; regression = 0.9434×10^6; SE = 0.157; p = 0.0001).

Over the time period of the meta-analysis, variation in laboratory practices, patient selection criterion, as well as expected inter lab variation in the individual results, weakens the validity of the meta-analysis but this data is non the less suggestive of a decline in semen concentration.

The other parameter reported on by Carlsen and her team was the mean seminal volume which also showed a negative temporal trend (Carlsen, et al., 1992). This result was less likely influenced by inter laboratory practices and is also indicative of a change in male reproductive function. The reported significant change in mean sperm volume was from 3.40 to 2.27ml (p = 0.027; SE = 0.0057) over the time period. Interestingly, these workers identified a geographic variation between the two Scandinavian countries of Finland and Denmark, with Denmark reporting lower sperm counts. Carlsen et al. (1992) also suggest a possible environmental aetiology for the changes in sperm quality and reproductive health. The credibility of the reports demonstrating a decline in male reproductive function in the academic press and lay media, primarily related to semen traits, was immediately questioned. These early meta-analysis also alluded to issues related to geographic variation in semen quality and potential causative factors but this was not the main focus of these researchers.

The scientific validity of Carlsen et al. (1992) was heavily criticised. The statistical methodology was questioned (Brake & Krause, 1992) as well as the credibility of laboratory techniques in the original research papers (Tummon & Mortimer, 1992). Ongoing criticism questioned all aspects of the research methodology, reviewing processes and reporting of this meta-analysis (Farrow, 1994). Alongside the critics there was some support for the suggestion of declining semen quality (Ginsburg & Hardiman, 1992) but there was no consensus.

Further empirical studies published after 1992 contributed to the weight of evidence suggesting a negative temporal trend in human semen quality. A number of French researchers responded in an attempt to identify temporal trends in semen quality. Although showing no change in seminal volume, a decrease in other parameters of sperm quality was seen over a 20 year period from 1973 in men attending a Paris fertility clinic (Auger et al., 1995). This publication supported the conclusions of Carlsen et al. (1992), but also corrected for age and sexual abstinence, with a decrease by year of birth in semen quality reported (-2.6%: concentration; -0.3%: motility; -0.7%: morphology / year of birth).

Another large study of French men (sperm counts: n = 19,848; men: n = 7714) demonstrated a decrease in semen quality (de Mouzon et al., 1996) with the most significant decrease seen in men born between 1950 and 1975. Another group of French researchers reported no change in semen quality but this was for the years 1977 – 1992 which, interestingly, do not include the main period of the previous research (1950 – 1975) where the greatest change in semen quality had been previously reported in French men.

A decline in fertility of men in Scotland was reported showing a lower concentration, total sperm output and motility in a later born birth cohort of men (Irvine et al., 1996). Similarly, a decline in semen quality was reported in a representative group of Belgian men (Van Waeleghem et al., 1996) but at this time Finnish men seemingly had no change in semen

quality (Vierula et al., 1996) nor did men in the United States (John, 1996). Contrastingly, semen quality in Greek men was seen to decline over a 17 year period from 1977 (Adamopoulos et al., 1996). Further research on Finnish men has subsequently contradicted the initial work on this nationality as it indicated a decline in semen quality from 1998 – 2006 (Virtanen et al., 2013).

Due to the importance of the debate on human fertility and male reproductive health, other researchers contributed to the discussion. A group, led by Shanna Swan, published the first of two meta-analyses (Swan et al., 1997). This was a reanalysis of the work of Carlsen et al (1992) and supported the hypothesis of an adverse temporal trend in human semen quality. Also Swan presented two other considerations in this meta-analysis. One was the difference between populations from the geographic regions identified (Europe, N. America, and Non Western). Secondly was an allusion to a potential environmental aetiology resulting in such a dramatic reduction in semen quality over a relatively brief time period. These workers presented the hypothesis, but came short of actually suggesting, a causative relationship between environment and reproductive health. Both of these factors would become prevalent in the further deliberations on reproductive health and fertility.

The work of Swan et al. (1997) contributed to the weight of evidence suggesting secular drift in human semen quality but did not squelch the debate. Antipodean researchers, in direct response to Carlsen's 1992 work, reported on the semen quality of 689 healthy men attending a fertility clinic in Sydney (Handelsman, 1997). These workers reported no significant change in semen quality during the period of 1980 – 1995. Interestingly, the magnitude of bias based on recruitment method was up to 100% which suggests that, if ignored, this methodological consideration is detrimental to the credibility of other reports on semen quality. Although in a different geographic region, contemporaries of these workers, reporting in the same year,

suggested no difference in semen quality by year of birth for men born in Denmark between 1950 – 1970 (Rasmussen et al., 1997).

A Canadian study concluded that during the time period of the study (1984 – 1996) that there was a downward trend in semen concentration (Younglai et al., 1998). These researchers also reported conflicting results dependant on the statistical method used. Another group of researchers, also working in North America (USA) and publishing in the same year, reported no change in semen quality (Emanueet et al, 1998). This group took the interesting approach of comparing previous research findings (MacLeod, 1951) with more current findings. When comparing these two similar groups at different time points, they found their contemporary group's (1971 – 1994) sperm count was not significantly different from the historic benchmark (1951) (102 ± 81 x 10^6/ml; 107 ± 74 x 10^6/ml respectively). It is difficult to directly compare the results of these two North American groups as they cover different time periods, with the Canadian information reported after the USA, and the variability in the results reported from the eleven Canadian centres (48.6 – 104.5 x 10^6/ml).

Several groups all reporting in 1999 found no change in semen quality (Andolz, 1999; Saidi et al., 1999; Tortolero, 1999; Zorn, Virant-Klun, Verdenik, & Meden-Vrtovec, 1999). Each of these reports concentrates on a single country (Spain, USA, Slovenia, Venezuela respectively) with variable results for semen concentrations. A comparison of these results would indicate that although no temporal decrease was reported in the individual countries a geographic variation in semen quality was demonstrated. Interestingly, the time periods did overlap in part or whole for all these studies (see Table 1.1 for an overview of related publication). This conflicting body of research initiated a second extended meta-analysis done by Swan and her group of researchers (Swan et al., 2000). They completed an additional meta-analysis of 101 research papers and again included the original 61 used by Carlsen (see Figure 1.1). This study

added to the weight of evidence and suggested a temporal decline in human male fertility. Due to the work of Carlsen (1992) and the validation of this by Swan (2000), there was a general acceptance that parameters relating to male fertility have shown a downward trend over the period 1930 – 1996 (see Figure 1.1) but following longitudinal studies continued to present conflicting results.

Men in Jeruselam, Israel were shown to have a decrease in semen quality (Almagor et al., 2003). During a ten year period from 1990 a decrease of 5.2×10^6 ($\pm 0.9 \times 10^6$ /ml) was shown in sperm count but it must be noted that the small group of men studied (n = 417) were from infertile relationships seeking fertility treatment. Another group of American men over a similar period (1989 – 2000) showed temporal trends in semen quality (Chen et al., 2003). No clear conclusion was drawn on overall change in semen quality but over the time period pH and motility increased while morphology declined. These workers also showed a positive relationship between birth year and semen volume, motility and concentration. Contradictory research from Australia showed no decrease in the semen quality with men presenting at a Melbourne fertility centre from two time periods (1977 – 1981; 1997 – 1998) (Clarke, Stewart, & Baker, 2004).

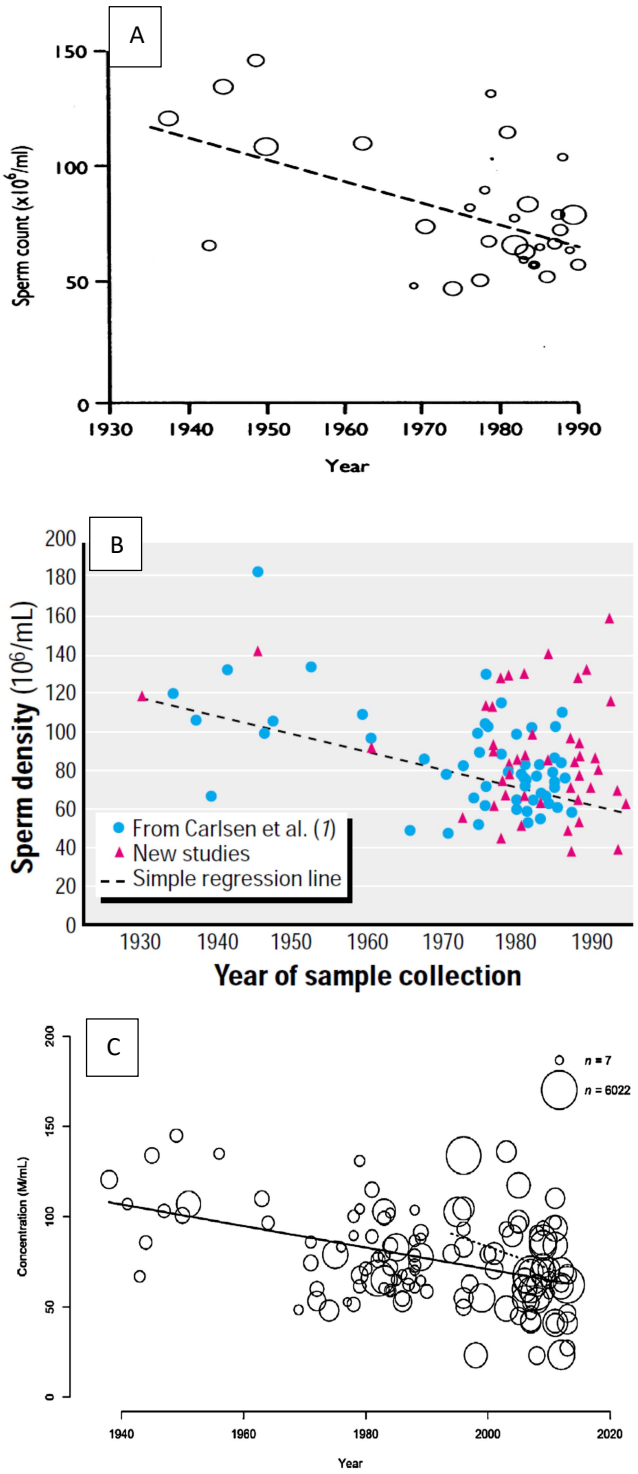


FIGURE 1-1 THREE SIGNIFICANT META-ANALYSES SUGGESTING NEGATIVE TEMPORAL TRENDS IN HUMAN SEMEN QUALITY. A) LINEAR REGRESSION OF MEAN SPERM DENSITY/CONCENTRATION (61 PUBLICATIONS) FROM 1938 - 1990. CIRCLE SIZES INDICATE WEIGHTING ACCORDING TO NUMBER OF SUBJECTS (CARLSEN, ET AL. 1992). B) LINEAR REGRESSION OF MEAN SPERM DENSITY/CONCENTRATION (101 PUBLICATION) FROM 1934 – 1996 (SWAN ET AL., 2000). C) SCATTER PLOT OF SPERM CONCENTRATION: 1938 – 2013(90 PUBLICATIONS). A MIXED EFFECT LINEAR MODEL WITH CIRCLE SIZES INDICATE WEIGHTING ACCORDING TO NUMBER OF SUBJECTS (JOHNSON, DUNLEAVY, GEMMELL, & NAKAGAWA, 2015)

The seemingly contradictory evidence presented by researchers is exemplified by two pieces of research by the same group in Japan. In 2007 this group presented work suggesting they

found Japanese men to have semen quality roughly equal to Danish men, who had near the lowest fertility in Europe (Iwamoto et al., 2007). Research published five years later concluded differently that Japanese men from four regions had semen quality similar to the best shown in European groups: Finland (Iwamotoal et al., 2013). Although the former research represented one area in metropolitan Tokyo with the later representing four areas, these conflicting results may be cautionary and represent methodological differences rather than secular trends.

Additional research globally suggested a change in semen quality. There was a significant decrease in a group of men in Tunisia (Feki et al., 2009). Men from two cities in New Zealand showed a decrease in certain parameters of semen quality over a twenty year period from 1987 (Shineet et al., 2008). More recent research showed some variation across geographic areas and a continued downward or levelling of the adverse trends. Axelsson et al. (2011) investigated sperm parameters in a group of Swedish men during the periods 2000 – 2001 and 2008 – 2010. They reported no significant difference in sperm parameters between the study periods. Another study, examining sperm parameters in a large group (n = 4867) of Danish men demonstrated an increase in median sperm concentration and total sperm count with no change in per cent motile and morphology during a 15 year period (1996 – 2010) (Jørgensen et al., 2012).

Additional research has demonstrated an adverse trend in male sperm quality with a significant decline in concentration and morphology in a large (n = 26 609) sample of men from across France (Rolland, et al., 2012). This is the largest single study to examine temporal trends in male fertility and the decrease of 32.2% over the study period (years 1989 – 2005) added weight to the argument that male fertility is declining. The work was supported by

another study of French men which also reported a decline in semen quality over the period of 1976 – 2009 (Splingart et al., 2012).

Men living in southern Spain were reported to have sperm quality in line with European men with median sperm concentration and total sperm count were 62 million/mL and 206 million/ml, respectively during the time period 2000 - 2001 (Fernandez et al., 2012). The research was used as an historic benchmark to compare semen collected and assessed in 2011 investigating temporal trends in semen quality (Mendiola et al., 2013). The suggestion, when comparing these two time points, is that there may be a decline in semen quality in young men in southern Spain. Over a similar period, men in Brazil also showed a decline in semen quality when comparing two time periods (2000 -2002 : 2010 – 2012) (Borges et. al., 2013).

Interest in male reproductive health, as represented by semen quality, has become the focus of researchers globally. In China, although some researchers suggest that there is insufficient data to draw conclusions on temporal trends in semen quality (Gu, 2014), one study representing a five year period (2007 -2012), suggested a decline in sperm concentration and normal morphology (Jiang et al., 2014). Although also representing a short time period of four years from 2010, corroborating research suggested a decrease in semen quality of students at a university in central China (Rao et al., 2015). Hardly conclusive due to the short time periods, these two studies followed methodologies as prescribed by the World Health Organisation (WHO) and intriguingly represent an emerging industrial economy. Both studies recognise the importance of ongoing research using semen quality as a proxy for reproductive and the overall health of Chinese men.

With the consideration of the confounding factors discussed in the literature since the publication of Carlsen's first meta-analysis in 1992, a critical review of the literature was

undertaken by one group and stated that there could be no scientific support for a decline in semen quality (Cocuzza & Esteves, 2014). In contradiction, an additional large scale meta-analysis extended the previous work of Swan et al. (2000), concentrating on 90 previously published studies with a main focus on age and semen quality. This work additionally included a longitudinal view of sperm concentration (Johnson et al., 2015). This group found that, over the time period examined (1938 – 2013) there was a decrease in semen concentration as a measure of semen quality (Figure 1.1). This would concur with and support the conclusions of the previous meta-analysis of both Carlsen et al, (1992) and Swan et al, (2000).

A solid conclusion or definitive interpretation of the body of evidence on temporal trends in semen quality is disconcerting. There can be no reassurance, especially in the early studies, of a standardized methodology, selection criteria, technical expertise or collection procedures. The need for standardised practices in the field of andrology and male fertility testing was recognised and met by the World Health Organisation's first published guidelines in 1980 (World Health Organisation, 1980) and with four subsequent editions up to 2010 (World Health Organisation, 2010). These guidelines demonstrated the requirement for standardisation of methodologies and as well as a necessity for updating these standards. Prior to these guidelines there is little evidence of standardised practices so early studies included in the historically based meta-analysis are implausible. Careful use of recognised methods and standardisation is required for reliable results when assessing semen quality (Mortimer et al., 1986) and therefore the historic data on semen quality must be viewed with caution (Tummon & Mortimer, 1992). This point has been made by many critics of large scale meta-analysis (Fisch, 2008; Jouannet et al., 2001; Pacey, 2013; Walczak-Jedrzejowska et al., 2013).

Other considerations, such as selection bias due to recruitment method, abstinence control and technical proficiency are a challenge when amalgamating research for the purpose of meta-analysis and for that reason should be removed or controlled for in the statistical model selected. For this reason and others, the statistical methodology in these meta-analyses has been heavily criticised (Brake & Krause, 1992; Younglai et al., 1998), specifically the use of linear regression modules on historic data related to semen quality (Farrow, 1994). When regression analysis was done on the same data used by Carlsen et al (1992) a completely different conclusion was reached with a suggestion of an actual increase in certain parameters of semen quality (Brake & Krause, 1992).

Sperm traits, or parameters as they are often termed in the literature, are a proxy for fertility and reproductive health but the answer to secular trends in quality of sperm may not to be found by meta-analysis (Ginsburg & Hardiman, 1992). A few overemphasised, peer reviewed articles, heavily cited by academic literature and reported in the lay press, may have created a false paradigm. The strong criticism of these meta-analyses must be considered along with the bulk of the research stating no decline in semen quality. A listing of the literature and statement of the summations of the research towards a decline or not is found in Table 1.1. Perhaps the only statement that can be made with confidence is that there is variation in semen quality and decline in specific geographic regions over specific time periods. Beyond secular trends in sperm quality, the story of male reproductive health extends to geographic variation and developmental perturbations. These additional proxy measures for fertility may provide more credible evidence of changes in male reproductive function and also provide insight into an aetiology.

TABLE 1-1 LIST OF SELECTED PUBLISHED WORK INVESTIGATING TEMPORAL TRENDS IN SEMEN QUALITY. THE CHRONOLOGICAL STARTING POINT BEING THE WORK OF CARLSEN ET AL (1992) WHICH INSTIGATED THE CONTEMPORARY DISCUSSION ON A TEMPORAL DECLINE IN SEMEN QUALITY.

Publication Year	1st author	Overall trends re semen quality	Study type	Period of study	Geographic Area	Reference
1992	Carlsen	Decrease	Meta-analysis (61 Studies)	1938 - 1991	Multiple countries	(Carlsen, Giwercman, Keiding, & Skakkebaek, 1992)
1992	Wittmaack & Shapiro	Unchanged	Empirical	1978 - 1987	USA	(Wittmaack & Shapiro, 1992)
1995	Auger	Decrease	Empirical	1973 - 1992	France	(Auger et al., 1995)
1995	Bujan	Unchanged	Empirical	1977 - 1992	France	(Bujan, Mansat, Pontonnier, & Mieusset, 1996)
1996	DeMouza	Decrease	Retrospective analysis		France	(de Mouzon et al., 1996)
1996	Adamopoulos	Decrease	Retrospective analysis	1977 - 1993	Greece	(Adamopoulos et al., 1996)
1996	Irvine	Decrease	Retrospective analysis	1984 - 1995	UK	(Irvine et al., 1996)
1996	Fisch	Unchanged	Empirical	1970 - 1994	USA	(Fisch et al., 1996)
1996	Van Waelegham	Decrease	Empirical	1977 - 1990	Belgium	(Van Waeleghem et al., 1996)
1996	Vierula	Unchanged	Empirical		Finland	(VIERULA et al., 1996)
1997	Rasmussen	Unchanged	Cohort	1950 - 1970	Denmark	(P. E. Rasmussen et al., 1997)
1997	Handelsman	Unchanged	Empirical	1980 - 1995	Australia	(Handelsman, 1997)
1998	Younglai	Decrease	Empirical	1984 - 1996	Canada	(Younglai et al., 1998)
1998	Emanuel	Unchanged	Empirical	1971 - 1994	USA	(Emanuel et al., 1998)
1999	Andolz	Unchanged	Retrospective analysis	1960 - 1996	Spain	(Andolz, 1999)
1999	Saidi	Unchanged	Meta-analysis (29 Studies)	1938 - 1996	USA	(SAIDI et al., 1999)
1999	Zorn	Unchanged	Retrospective analysis	1983 - 1996	Slovenia	(ZORN et al., 1999)
1999	Tortolero	Unchanged	Empirical	1981 - 1995	Venezuela	(TORTOLERO, 1999)

2000	Swan	Decrease	Meta-analysis (101 Studies)	1934 - 1996	Multiple countries	(Swan, Elkin, & Fenster, 2000)
2003	Almagor	Decrease	Retrospective longitudinal	1990 1999	Israel	(ALMAGOR et al., 2003)
2003	Chen	Temporal trends	Empirical	1989 - 2000	USA	(CHEN et al., 2003)
2004	Clarke	Unchanged	Retrospective analysis	1971 - 1998	Australia	(Clarke et al., 2004)
2005	Carlsen	Unchanged	Prospective	1997 - 2001	Denmark	(Carlsen, Swan, Petersen, & Skakkebaek, 2005)
2005	Lackner	Decline	Retrospective	1986 - 2003	Austria	(Lackner et al., 2005)
2008	Shine	Decrease	Empirical	1987 - 2007	New Zealand	(Shine et al., 2008)
2009	Feki	Decrease	Empirical	1996 - 2007	Tunisia	(Feki et al., 2009)
2011	Axelsson	Unchanged	Historical	1940 - 1990	Sweden	(Axelsson, Rylander, Rignell-Hydbom, & Giwercman, 2011)
2012	Splingart	Decrease	Population	1976 - 2009	France	(Splingart et al., 2012)
2012	Rolland	Decrease	Retrospective analysis	1989 - 2005	France	(Rolland et al., 2012)
2012	Jorgenson	Increase	Cross sectional population based	1996 – 2010	Denmark	(Jorgensen, 2002)
2013	Virtanen	Decrease	Decrease	1998 - 2006	Finland	(Virtanen et al., 2013)
2013	Mendiola	Decrease	Retrospective analysis	2002 - 2011	Spain	(Mendiola et al., 2013)
2013	Borges	Decrease	Longitudinal cohort analysis	2002 - 2012	Brazil	(Borges et al., 2012)
2014	Jiange	Decrease	Cohort	2007 - 2012	South West China	(Jiang et al., 2014)
2014	Cocozza	Unchanged	Systematic Review	1995 - 2012	Multiple countries	(Cocuzza & Esteves, 2014)
2015	Johnson	Decrease	Meta-Analysis (90 Studies)	1938 - 2013	Multiple countries	(S. L. Johnson et al., 2015)
2015	Rao	Decrease	Four year longitudinal	2010 - 2013	Central China	(Rao et al., 2015)
2015	Borges	Decrease	Retrospective cohort study	2000 - 2012	Brazil	(Borges,et al., 2015)

1.3 GEOGRAPHIC VARIATION IN HUMAN SEMEN QUALITY

Perhaps initially a serendipitous finding, resulting from the research investigating temporal trend in semen quality, variation in sperm parameters by geographic region is a more credibly reported phenomenon. This credibility comes from research designed specifically to investigate this hypothesis with less reliance on retrospective meta-analyses.

The variation in semen quality across different geographic locations was examined in a large scale prospective study and variation in different European locations was demonstrated (Jorgensen, 2001). This work reported on parameters related to male fertility using semen samples of men from four European cities. The methodologies, across the four laboratories used coordinated standardised procedures following World Health Organisation (1992) protocols. The quality control and quality management was confirmed across the participating labs and no significant difference was reported. They reported variation in sperm concentrations and counts. Danish men (Copenhagen) reported the lowest semen quality with French, Scottish and Finish men demonstrating respective increases in semen quality (Jorgensen, 2001).

A follow on study (Jorgensen, 2002) examined semen quality in four northern European countries and again found regional differences. In a group of young men (n = 968) from the general population attending compulsory medical examination, adjusted median sperm concentration ranged from 57 million/ml (Estonia) to 41 million/ml (Norway and Denmark) (Jorgensen, 2002). The researchers suggested an east west gradient in semen quality with Finnish and Estonian men having higher quality semen than those from Denmark and Norway.

Men from Finland in this study had an adjusted median sperm concentration of 54 million/ml which validates the findings of the previous research which reported men from Denmark having poorer sperm quantity when compared to men from Finland. Although much emphasis was placed on the seemingly different semen quality between Danish and Finnish men, this must be considered in light of later research which demonstrated a decrease in Finland to a level of semen quality matching those of Danish men (Virtanen et al., 2013).

Previous studies had excluded Sweden in allowing a comparison across Scandinavian countries. This comparison of a distinct culturo-linguistic region could provide insight into a lifestyle or environmental aetiology. Semen quality data was collected from healthy young Swedish men (n = 305) undergoing compulsory medical examination (Richthoff, 2002). These values, compared men in the sample group born in Sweden to those collected by Andersen (2000) representing Finnish men and demonstrated significantly better semen quality in Swedish men. Confounders taking into consideration, the conclusion of these workers was that two genetically similar populations showing differences in reproductive health could be explained by lifestyle and, more interestingly, environmental factors.

Other studies using men from the Baltic regions showed higher semen concentrations in Lithuanian men when compared to Estonian men from the general population (Punab et al., 2002). This work also reported higher concentrations in the Estonian group (67×10^6 / ml) to those reported previously (57×10^6 /ml(Jorgensen, 2001)). Although it cannot be ignored that these results used different selection criteria, the samples collected three years later than Jogenson's group did not have confirmed QC/QM in line with the original laboratories.

A seminal investigation in 2006 (Jørgensen et al., 2006) confirmed the geographic variation in semen quality in men from Baltic and Scandinavian countries. Denmark and Norway showed poorer semen quality (median 41 x 10^6 /ml) than did Finland (median 54 x 106 /ml) and the other countries.

Similar observations have demonstrated significant geographic variation in male fertility in North America (Swan et al., 2002). A prospective study was designed in collaboration with the multicentre European study of Jorgensen et al. (2001) so that credible and direct comparisons could be made. The four study centres (Concentration: New York = 58.2, Missouri = 35.0, Los Angeles = 43.4 , Minnesota = 54.9 (10^6 / ml)) showed geographic variation in certain semen traits including volume, motility, morphology as well as concentration. A greater geographic variation in concentration to that which was seen in the European study was also reported. Each of the 8 centres represented by the European and the American studies all reported differences in semen quality. This is a strong indication of geographic variation in semen quality but this phenomenon remains idiopathic.

The body of work which examined geographic variation in sperm traits in consideration of the criticism of the previous studies on time-trends on semen quality, created more credible data that could be used as benchmark values for further investigations. As an example of this, one study using previously reported data showed Greenland had the lowest median sperm concentration (53 x 10^6 / ml) of any of the geographic and national regions in Europe or America (Toft, Pedersen, & Bonde, 2004). This further supports research reporting geographic variation in semen quality but this work also demonstrated intra national differences in certain semen traits. Men from eastern Greenland, worth noting as an area of higher serum concentrations of organochlorides (Deutch & Hansen, 2000), showed lower volume and

motility when compared to other areas in the same country and suggesting a link to a more traditional diet.

Further support for geographic variation in semen quality came from a Chinese study which demonstrated variability by both geographic regions and land use ((urban vs. rural) Gao et al., 2006). Additionally, Southwest China reported differences in semen quality when compared to other areas of China, USA and Europe (Gu, 2014). In support of this work other Asian researchers showed small but significant differences in four regions across Japan (Iwamoto, Nozawa, Mieno, et al., 2013).

In order to determine geographic differences between two distinct areas of Germany, samples donated by men from Leipzig (former West Germany) and Hamburg (former East Germany) were compared for semen quality (Paasch et al., 2008). No significant difference in these groups was found for concentration but there was for morphology and motility. Of more concern in these two groups was the overall low quality of the semen with mean concentrations below what is reported to reduce fertility. The low quality of the semen reported compared to that found in men from Denmark and Norway (Jorgensen, 2002). This may indicate that sub optimal semen quality may be a European wide rather than a Scandinavian phenomenon. Changes in semen quality are only one aspect of fertility which has been reported as having negative secular trends. Other developmental disorders have also been examined for similar trends as reviewed in the next section.

1.4 TRENDS IN THE INCIDENCE RATE OF TESTIS CANCER

The WHO, through the International Agency for Research on Cancer, collects and publishes data on cancer incidence rates worldwide (website - http://ci5.iarc.fr). This organisation has stated the worldwide occurrence rate of cancer of the testes is 0.7% of all major cancers with an age-world standardized incidence rate of 1.5 (per 100 000 men). Although having a low occurrence rate, testes cancers, as this is an umbrella term, is of concern because it is increasing in incidence and is a cancer of young men (seminomas predominate in 15 -19 year olds (Liu et al., 2000)).

There is a large body of epidemiological and other evidence demonstrating adverse temporal trends in testes cancers (Cotterill et al., 2000; Liu et al., 2000; Moller, 2001; Toledano et al., 2001; Huyghe et al., 2003; Richiardi et al., 2004; Cooper et al., 2005; Bray et al., 2006; Walsh et al., 2006; L'esperance et al., 2008; Walschaerts et al., 2008; Lacerda et al., 2009; Cocuzza & Esteves, 2014). These numerous reports indicated overall negative trends showing a birth cohort effect but a levelling in developed countries and of increase in developing countries.

The reported difference in the occurrence and incidence of testes cancers between geographic regions and the association between geographic regions is worth reviewing. Western economic regions have a higher rate of testicular seminomas (Bray et al., 2006) although this increase has abated. Specifically, Denmark has a higher rate of seminomas than Finland (10.1 vs 4.5 per 10000 of population) during the time period of 2003 – 2007 with Norway reporting the highest rate (10.5) and Estonia the lowest (2.8) (Trabert et al., 2015). These results clearly demonstrate variation in seminomas and nonseminomas by country in Europe.

Developing economic regions are seeing an increase in testes cancers. Differences in the incidence rates between ethnic groups have been reported in the USA. White men have a

reported incidence rate of 9.9 vs black men reporting a rate of 1.4 when looking at a birth to 19yo group between 2006 – 2010 (Ward et al., 2014). Other researchers have reported an incidence rate of 5.1 overall with 6.3 and 1.1 for white and black ethnic groups respectively (Townsend et al., 2010) base on seminomas, nonseminomas and germ cell cancers. But the negative trends have attenuated with later birth cohorts in developed countries with stabilisation of USA populations between 1999 – 2004 (Townsend et al., 2010).

The increased incidence of testis cancer, described as rapid (Znaor et al., Lortet-Tieulent et al., 2015), has shown adverse trends in the last 60 – 70 years (Richiardi et al., 2004). There has been a doubling of the incidence rate in the last number of decades (Huyghe et al., 2003; Walschaerts et al., 2008) with an estimate of 1% per year increase in Europe between 2005 – 2025 with and overall 24% increase in this time period based on models using retrospective analysis (Znaor et al., 2015). A large (n = 27 000) epidemiological study, in addition to reporting an overall increase in the rate of testis cancer, showed variation across 8 different geographic centres. In another study on global trends related to the incidence of testis cancer and mortality, a great variation was reported across geographic areas with the highest rates reported in Caucasian populations living in developed nations (Rosen, et al., 2011).

Epidemiological data showing an increase in the rate of and geographic variation in the incidence of testes cancers is credible evidence of changes occurring in male reproductive health. This data, although not irrefutable, does not lend itself to the criticism of methods seen with the early meta-analysis showing temporal trends in sperm quality. But like the theorised negative trends in sperm quality, the time period is suggestive of an aetiology linked to environmental or lifestyle changes.

1.5 Trends in the Incidence Rate of Cryptorchidism

Like testes cancer rates, the incidence of cryptorchidism seems to be increasing. Also like cancer rates, these changes are happening over a time scale which does not support a genetic aetiology. Congenital cryptorchidism is defined as non-descended testis at birth (Priskorn et al., 2012). Presenting as one or more of the testes not descending into the scrotum (Paulozzi, 1999), the aetiology of cryptorchidism is multifactorial and could be endocrinological, neurological, mechanical or other acquired abnormalities (Hutson et al., 2013). There are also classifications based on location along the normal line of decent (abdominal, inguinal canal, external inguinal ring, pre or upper scrotum) or as ectopic; also most often unilateral (= 77% of cases (Ferguson & Agoulnik, 2013)) (usually right sided) or bilateral (Ayhan et al., 2013).

For a variety of reasons the diagnosis and reporting of cryptorchidism may not truly reflect the incidence rate and new-borns must be distinguished from children as 50% of cryptorchid testes descend by 1 month (Chul Kim et al., 2011). Generally the global rate is reported as 2-4% of all boys (Barthold & González, 2003) but higher rates of 2-9% of new-borns affected have also been reported (Toppari et al., 2010). As with the other aspects of reproductive health and fertility, there seems to be geographic differences in the incidence rate of cryptorchidism (Priskorn et al, 2012). In parallel to reported differences in semen quality between Finland and Denmark (Jensen, 2000) a significant difference in cryptorchidism has been reported between Finnish new-borns (2.4%) and Danish new-borns (9.0%) (Boisen et al., 2004). This research also reported that Denmark had a significantly higher rate of cryptorchidism than 40 years ago.

Other countries have also reported rates of cryptorchidism. The UK occurrence rate of 5.9% (Acerini et al., 2009) falls between Denmark and Finland. Hungary has had no change in the last 50 years (Mavrogenis et al., 2015). Korea has a very high incidence rate of 17.43 per 10 000 births in 2005 (Chul Kim et al., 2011).

The overall conclusions would be that there has been an increase in the incidence rate and this varies by country. This conclusion must also be made in consideration of some of the reports which seem contradictory but the overall weight of evidence suggest an increase in the incidence and geographic variation. The underlying causes of cryptorchidism have been linked to the environment and this theory is supported by the time scale of the reported increase and the geographic variance.

1.6 TRENDS IN THE INCIDENCE RATE OF HYPOSPADIAS

Hypospadias is one of the most common developmental malformation of human male infants and importantly for consideration in the context of this thesis, hypospadias is strongly associated with endocrine disruptions during foetal development. It has been defined as a midline fusion defect of the male urethra which results in a misplaced urethral meatus (Kalfa et al., 2009). Similarly but more specifically, the European Association of Urology defines hypospadias as hypoplasia of the tissues forming the ventral aspect of the penis beyond the division of the corpus spongiosum. This congenital malformation results from abnormal joining or fusion along the ventral midshaft seam of the urethra during foetal organogenesis (Baskin et al., 2001). The location of the ectopic urethral opening in proximity to the external meatus and pelvic bone determines the classification. Three general categories exist: mild (effecting the glandular region), moderate (coronal, distal penile, midshaft, proximal penile regions), severe (penoscrotal, perineal regions) (Palmsten & Chambers, 2015).

Although temporal trends are difficult to determine, an increase has been suggested in the incidence rates reported both in North America and Europe over the past 50 years (Paulozzi, 1999). Research has confirmed the increase in the incidence rate of hypospadias but also suggest it is localised by geographic region (Carmichael, et al., 2012). All European congenital abnormalities are currently registered with EUROCAT statistical monitoring and the monitoring of hypospadias has shown adverse temporal trends in 4 million births between 1999 – 2008 (Loane et al., 2011). This Pan-European data showing an increase in hypospadias is supported by research from other countries(Paulozzi et al., 1997; Paulozzi, 1999; Aho et al., 2000; Sun, Tang et al., 2009; Chul Kim et al., 2011; Fisch et al., 2010; M. S. Jensen et al., 2012;).

Additionally research in Western Australia demonstrated a significant increase (approximately 2% per annum) in the incidence of hypospadias between 1980 – 2000 (Nassar et al., 2007).

Similarly, researchers in Sweden reported an increase between 1990 -1999 in a study covering a 40 year period from 1972 (Nordenvall et al., 2014). This was supported by Chinese researchers who also reported an increase over a ten year period from 1998 (Sun et al., 2009) and additional work showed an annual increase of 7.3% (1996 – 2008) with the highest increase of 9.08% found in rural areas (Li et al., 2012) for this country.

There is however a number of opposing theories put forward with Finnish boys having no change in the incidence rate of hypospadias between 1970 – 1994 (Aho et al., 2000). Additionally, reports from specific regions of the USA have not indicated increasing secular trends in hypospadias. A group of boys (n = 5838) born in California between 1984 – 1997 showed no increase in incidence. Two other regions of the USA have reported no change. A population based study showed no increase in hypospadias in Washington state (Porter, Faizan et al., 2005) and in New York no changes have been demonstrated between 1992 – 2005 (Fisch et al., 2009).

Not surprisingly some studies inevitably contradict reports of a temporal increase in boys born with hypospadias. What the weight of evidence does suggest is that there would seem to be, in some geographic areas over certain times, a negative trend showing an increase in the incidence rate of cryptorchidism.

Sperm quality, cryptorchidism, testes cancer and hypospadias are all diseases or conditions of the male reproductive tract which are all reported as showing negative secular trends. Considered individually, absolute conclusions regarding these trends would be untenable. The power of the argument that specific parameters of male reproductive function are adversely affected comes from considering the comorbidity of these conditions.

1.7 Reproductive Health and Testicular Dysgenesis Syndrome

The widely reported adverse temporal trends in semen quality may be an artefactual phenomenon and is contested in some of the literature. More robust epidemiological data is available on other aspects of reproductive male health such as cryptorchidism, hypospadias and testes germ cell cancer. Testes cancer provides the strongest evidence for changing male reproductive health as the epidemiological data suggesting increased rates of testes cancer is more robust and credible. It is also accepted that cryptorchidism is a risk factors for testes cancer (Ferguson & Agoulnik, 2013) as boys with cryptorchidism are 3x more at risk for testes cancer (Lip et al., 2013). As putative risk factors for each other, a link between testes cancer and maldevelopment is credible as these disorders are antenatal in origin. It has been suggested that testes cancer is the underpinning condition to all these adverse reproductive health problems and the likelihood of testes cancer increases with each incident of one other conditions (Bay et al., 2006). Additionally, testes cancer has been linked to poor semen quality (Carlsen et al., 1995; Jacobsen, 2000)

A large cohort study (n = 2 159 883) of Danish men did not support the hypothesis that cryptorchidism, hypospadias and testes cancer had common heritability but did show an association overall between the risk of hypospadias/cryptorchidism and testicular germ cell cancers (Schnack et al., 2010). In agreement with this research it has been suggested by other workers that cryptorchidism and hypospadias have a partly shared aetiology (Akre et al., 1999).

The logical extension of this argument is the suggestion of a link between the four disorders of male reproductive health demonstrating adverse trends. Therefore, four conditions of perturbed reproductive development would make up a Testicular Dysgenesis Syndrome (TDS)

(Skakkebaek et al., 2001). In support of this hypothesis, evidence that each of these disorders is a risk factor for the other has been put forward (Sharpe & Skakkebaek, 2008). For example, testicular maldelvelopment is is reported to be significantly correlated with testis cancer (Skakkebaek, et al., 2001). Therefore a reduction in semen quality cannot be considered in isolation as these conditions have all been suggested as having one underlying pathogenesis (Bay et al., 2006). Therefore, due to the temporal trends in semen quality, testis cancer, hypospadias and cryptorchidism, combined with the suggested common aetiology and interrelationship, it is not surprising that it has been hypothesised that they comprise a Testicular Dysgenesis Syndrome (TDS) (Skakkebaek et al., 2001; Bay et al., 2006; Sharpe & Skakkebaek, 2008).

Anogenital Distance (AGD) is not included in the suite of reproductive disorders which make up TDS but is suggested as a bioassay for reproductive maldevelopment and foetal androgen dependant processes (Salazar-Martinez et al., 2004). TDS has been strongly linked to androgen exposure during a sensitive Male Programming Window defined in rodent studies (Welsh, et al., 2009). In mechanistic animal studies the measurement of anogenital distance has become routine when examining effects of EDs on reproductive development. More specifically, as male reproductive development, especially of the perineal area, is dependent on dihydrotestosterone (Bowman, et al., 2003), perturbations of androgen concentrations will alter AGD in rats. The importance of AGD as a marker for human reproductive development has been studied (Salazar-Martinez, 2004) and there is a suggestion that shorter anogenital distance is related to poorer sperm quality (Mendiola, et al., 2011). This group also recommended further research into AGD as a biometric for developmental androgen concentrations and thus a predictor of TDS. There could also be an argument for including shortened AGD as a fifth factor in the suite of conditions making up TDS.

The theory of a human TDS is well supported by workers in the field of andrology and reproductive biology. This theory is supported by a body of literature which persuasively argues that sperm quality is decreasing. Stronger evidence comes from the epidemiological research suggesting increases in male reproductive maldevelopment and cancer. The suggestion of an environmental aetiology for human TDS is supported by the short time period in which TDS has manifest itself. If an environmental cause is a plausible explanation then other animals which share our environment may provide substantiation for this hypothesis.

1.8 SUPPORT FOR ADVERSE FERTILITY TRENDS IN NON-HUMAN SPECIES

A decline, similar to that seen in human male fertility, has also been reported in a range of other species. The endangered Florida panther has shown a dramatic decline in reproductive health and fertility. Alarmingly, the per cent of male offspring with undescended testes increased exponentially from 1975 up to the time of reporting for the study (Facemire et al.,1995). Similarly, an investigation designed to evaluate temporal trends in semen quality in a large group of dairy bulls has identified an overall decline in fertility in a thirty year period from 1965 (Wahl & Reif, 2009). It is worth noting that although the overall trend showed a decrease in total sperm output, from 1985 to 1995 showed an increase from the lowest reported mean value 1985). Although as with the human data, there has been some contradictory research with another research group reporting that between the years 1932 – 1995 there was no change in sperm concentration in semen of bulls (Setchell, 1997). This same work of Setchel (1997), which was a meta-analysis of the relevant research for the period, also reported no significant change in boar semen and a significant although slight, increase in ram semen concentration.

Direct comparisons with human studies must be done with caution as production animals are indirectly selected for fertility. This confounding factor, although recognised in this meta-analysis, is largely discounted. Subsequent reviews recognise the complexity of environmental exposures and realise that pollutants have potential major implications for the reproductive potential of production animals (Sweeney, 2002). The hypothesis that environmental exposures could threaten farm animal productivity is further supported by other researchers (Rhind, 2005; Petro et al., 2010).

Even more alarming are the developmental malformations and decrease in fecundity recently seen in wildlife species. A small group of St. Lawrence Beluga Whales are showing symptoms of immunosuppression, endocrine dysfunction and hermaphroditism (Deguise, et al., 1994). Reproductive failure is suspected in the decline of other marine mammals, such as seals, California sea lions and the Dall's porpoise of the northern Pacific. Artic Polar Bears living in remote locations have also been identified as developing pseudo-hermaphroditism (Carmichael, et al., 2002). In Florida, USA, male alligators living in Lake Apopka had a reported 24% mean reduction in the size of their penis compared to alligator populations in other Florida lakes. This was one manifestation of a crash in the population in these reptiles and was traced back to contamination of the lake several year before (Guillette et al., 1985). In addition to these there are other examples of declining fertility occurring over a similar time frame to that observed in the human. These examples of changes in fertility and reproductive disorders found in wildlife and other non-human species are strongly indicative of an environmental aetiology.

The evidence of perturbations to reproductive function and development in wildlife, as reported in brief above, is indicative of a decline in fertility similar to that reported in humans. The negative changes in sperm quality and reproductive malformations seen in animals when considered against the parallel changes in humans suggest a common aetiology. The reproductive changes reported in wildlife are generally attributed to environment pollutants leading logically to the suggestion of the same aetiology in humans.

1.9 ENDOCRINE DISRUPTORS AS AN ENVIRONMENTAL FACTOR IN ADVERSE TEMPORAL TRENDS.

The theory that there has been over the past 50 -60 years adverse trends in male reproductive health, largely accepted although emotive, is of concern to both the general public and scientific community. The short time frame of these adverse trends and the variation across geographic/national boundaries are not suggestive of genomic evolutionary mechanisms but of an environmental or lifestyle aetiology (Pinto & Carvalho, 2013; Woodruff, 2011; Balabanič et al., 2011). In consideration of this, many researchers have suggested that a group of chemicals, collectively termed endocrine disruptors (ED), endocrine disrupting compounds or chemicals (ED) are associated with changes in reproductive health (Majdič, 2010; Pflieger-Bruss et al., 2004; Priskorn et al., 2012). For the past thirty-five years, there is increasing evidence of a connection between harmful environmental compounds and reduced male fertility. More recently, there have been reports of an association between ED exposure and reproductive problems in women e.g, polycystic ovarian syndrome, recurrent miscarriage and precocious puberty in developing girls (Zama & Uzumcu, 2010). These pathologies have been collectively termed Ovarian Dysgenesis Syndrome (Fowler et al., 2011) suggesting a parallel syndrome to TDS.

Many of the suspected compounds (e.g., Phthalates, Polychlorinated biphenyls (PCB), polybrominated diphenyl ethers, polyfluorinated ethers, bisphenol A, polycyclic aromatic hydrocarbons) are ubiquitous in the environment and either act like hormones or inhibit the normal action of hormones. Due to their ability to disrupt normal endocrine function and subsequent development these substances act via the endocrine system.

There have been numerous definitions and descriptions put forward for these substances and some of these are outlined in Table 1.2. A satisfactory definition must include the fact of an ill health endpoint to differentiate chemicals which may perturb the endocrine system but not detrimentally. The exact quantification of the suggested ill health endpoint may be difficult for certain suspected EDs but this factor needs consideration. A working definition for the purposes of the proposed research would be: An endocrine disruptor is a ubiquitous, exogenous, anthropogenic environmental chemical or compound that alters the function(s) of the endocrine system and consequently has a suspected or proven adverse health endpoint in an intact organism, or its progeny. It should also be emphasised that EDs, unlike other toxicants, can be effective at what has been termed 'low dose' (Vandenberg et al., 2012).

TABLE 1.2EXAMPLES OF DEFINITIONS AND DESCRIPTIONS OF ED AND ENDOCRINE DISRUPTION

Definition and ED and endocrine disruption	Reference
…natural and synthetic chemicals can interfere with the normal functioning of the endocrine system, thus affecting reproduction and development in wildlife and humans	Nollet, 2011
Endocrine disrupting chemicals are today considered as a varied group of chemicals that include certain pesticides (DDT, organophosphorous pesticides, possibly atrazine, polychlorinated and polybromated biphenyls, PCBs andPBBs), plasticizers (octylphenol, nonylphenol nonylphenol), plastic components (phthalates, bisphenol A) and also some naturally occurring estrogenic compounds, so called phytoestrogens, such as coumestrol and genistin.	(Majdič, 2010)
Environmental oestrogenic and anti-androgenic compounds	(Skakkenbaek et al, 2001)
Endocrine disrupting compounds (EDs) comprise a range of anthropogenic chemicals, including polychlorinated biphenyls(PCBs), polybrominated diphenyl ethers (PBDEs) and polycyclic aromatic hydrocarbons (PAHs), all of which are highly persistent and remain in the environment for periods of years (Smith, 1996), and phthalates and alkyl phenols which are more readily degraded but are also continually produced and released into the environment (Clark et al., 1992; ECPI, 1995).	(Rhind et al., 2010)
…interfere with the normal function of the endocrine system.	(Ranjit, Siert and Padmanabhan, 2010)
…act as either hormone agonists or antagonists or the ability to disrupt hormone synthesis, storage or metabolism.	(Sweeney,Torres, 2002)
Endocrine disrupting chemicals (EDs) are chemicals that can have an effect on the endocrine system of animals including humans.	(Singhal et al, 2010)
...endocrine disruption is the insidious trespass of man-made chemicals into every vital organ system in your body that comprises or is controlled by the endocrine system, such as the thyroid and parathyroid, pancreas, adrenals, thymus, male and female reproductive organs, the heart, digestive system, and skeletal system -- all the systems that participated in how you were constructed in the womb and how you are functioning today	Colburn, 2010
… they interfere with the action of endogenous hormones.	(Rudel & Perovich, 2009)
… from a physiological perspective, and endocrine-disrupting substance is a compound, either natural of synthetic, which, through environmental or inappropriate developmental exposures, alters the hormonal and homeostatic systems that enable an organism to communicate with and respond to its environment	(Diamanti-Kandarakis et al., 2009)
Endocrine disruptors have been defined as exogenous substances that alter function(s) of the endocrine system and consequently cause adverse health effects in an intact organism, or its progeny, or subpopulations.	WHO Internal Programme for Chemical Safety.

The chemicals found in the environment which fit the definition above are numerous. The Commission of European Communities (2007) suggested that there are 320 chemicals in this category. The Endocrine Exchange (TEDEX), an organisation founded by the late Theo Colburn (a pioneering worker in this area), has at the time of writing, a data base of 1583 chemicals or compounds having at least one peer reviewed research publication that demonstrates endocrine disrupting properties. These numbers are not definitive as there are new compounds being continually proposed as endocrine disruptors (Kortenkamp, 2007). A more conservative and credible claim of over 800 man-made chemicals known to disrupt the endocrine system is put forward by UNEP and WHO (WHO, 2013).

The Commission on Research Action group of the European Commission, in their 2005 report 'Tackling the Hormone Disrupting Chemical Issue', put forward two broad classifications of EDs: synthetically produced hormones and man-made chemicals. The Endocrine Society more specifically uses the categories of industrial solvents/lubricants, plastics, plasticisers, pesticides, fungicides, pharmaceutical agents (Diamanti-Kandarakis et al., 2009). The difficulty in categorising EDs becomes evident as it is now accepted that certain defoliants, phytoestrogens (Nohynek, et al., 2013) as well as personal care products are also suspected EDs.

As a broad group of chemicals found in the environment, the pathways of exposure differs. It is generally accepted that routes of contamination are dermal, intestinal, respiratory by either touch, ingestion or inhalation. Figure 1.2 below shows the typical pathways of contamination in humans of a lipophilic bioaccumulating ED.

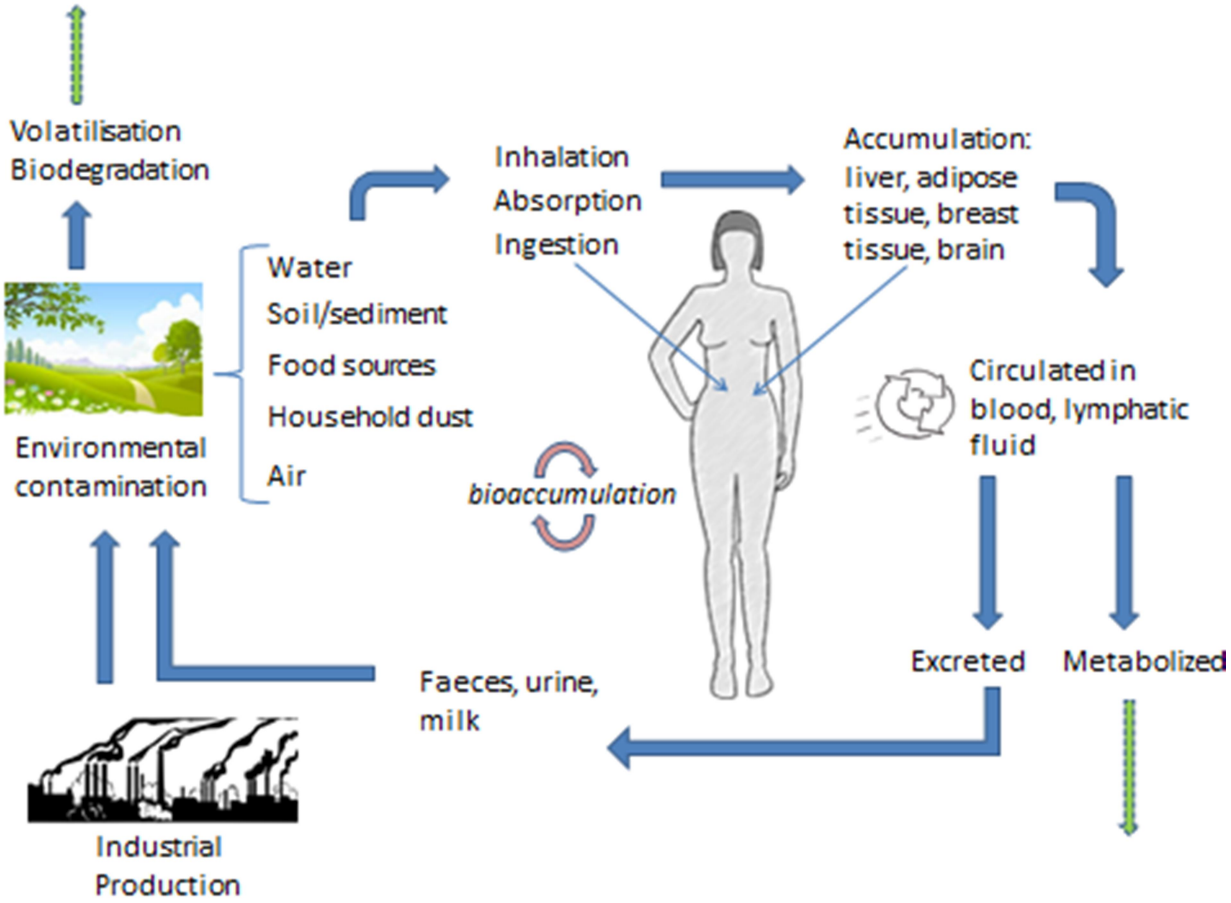


FIGURE 1-2 TYPICAL PATHWAY AND ROUTES OF CONTAMINATION FOR A LIPOPHILIC ED CHEMICAL. BLUE ARROWS INDICATE PATHWAY OF CHEMICAL AND CIRCULATION WITH GREEN ARROWS INDICATING THE REMOVAL OF THE INTACT CHEMICAL OR METABOLITE FROM THE CYCLE. BIOACCUMULATION IS INDICATED BY RED SHADING AS A SEPARATE PATHWAY AND CIRCULATORY ROUTE FOR THIS PHENOMENON RELATED TO ED EXPOSURE AND CONTAMINATION.

Of the many hundreds of chemicals and compounds theorised and proven to have endocrine disruptive effects, resources often limit individual research projects to the selection of small numbers of representative chemicals. Three chemicals which fit the definition of an endocrine disruptor and are also found on the list put forward by WHO and the Endocrine Exchange are PCBs, PBDEs and DEHP. As typical EDs, these chemical groups are found in tissue and body fluids (Gray, 2000; Law et al., 2014; Petro et al., 2010). The routes of contamination of EDs are generally via ingestion, absorption of inhalation and the substance will either be stored or metabolised. Due to its lipophilic nature a chemical like PCB can be stored in adipose tissue for

up to five years (Phillips et al., 2010) and perhaps longer as the similar chemical Dioxin has a half-life in humans of approximately seven years (Hoogenboom et al.,2015).

Alternatively, BPA and phthalates are readily metabolised and excreted showing a short biological half-life (Rudel et al., 2011). As half-life values are metabolism dependent, it is likely that it is shorter in children and longer in adults.

Recognised as an exemplar ED, Polychlorinated biphenyls (PCBs) are a group of chemicals, consisting of 209 congeners, which have been extensively examined in relation to their endocrine disrupting properties. Of the groups of industrial chemicals, PCBs are the most extensively used in the field of research. These chemicals, classified as organohalogens, have been produced in large quantities from 1929 until being banned, as a probable carcinogen, in Canada (in 1977 (Health Canada, 2015)) and the USA in 1979 (EPA, 2015) as well as their use being heavily restricted in Europe since 1985 (European Commission, 2015). Due to their stability and heat resistance these chemicals had many industrial uses and historic global production is conservatively estimated at 1.3 million tonnes (Breivik et al., 2002). The legacy of PCB, due to its stability, will remain in the worldwide environment and all its organisms indefinitely.

PCB congener mixes, Araclor being the most common (EPA, 2015), and single congeners both had industrial uses. The single congeners are identified by their congener number and commonly seven of these have been used to monitor concentrations in the environment. These congeners (ICES 7 PCBs = congeners 28, 52, 101, 118, 138, 153, 180) have been identified by the International Council for the Exploration of the Sea (ICES) as the ones found in the highest concentrations in fatty fish (Webster, et al., 2013) and have been recognised as reference congeners for environmental monitoring by OSPAR commission Co-ordinated Environmental Monitoring programme (CEMP).

1.10 Plasticisers As Endocrine Disruptors

The endocrine disrupting effects of plastic and chemicals used in their manufacturing process have received media attention based on research reporting that most of these substances interfere with endocrine function (Yang et al., 2011). The most well-known plasticiser from the phenyl group of chemicals, Biphenyl A (BPA), is found in epoxy resins (often used to line food containers) and poly carbonate plastics. The global production capacity was reported as 4.7 x 10^6 tonnes (Jiao et al., 2008) with production increasing by over 4% annually in the USA (Huang et al., 2012). Humans are exposed to BPA on a daily basis (Kang, Kondo, & Katayama, 2006) due to its countless domestic uses which have created a ubiquity in the environment resulting in 92.6% of people over six years of age having detectable somatic concentrations of BPA (Trasande et al.,2012). There is strong evidence that BPA has an estrogenic effect on humans (vom Saal & Welshons, 2014) and other animals (vom Saal et al. 2007).

Another group of plasticisers, phthalates or phthalate esters have many uses including personal care products, thermal inks, pharmaceuticals, furniture and clothing, pesticides and building materials (Niraj Pant et al., 2008). Phthalates, at 18 billion pounds per annum, has the highest annual production of any chemical type (Latini et al., 2004), and are the most commonly used plasticiser. Polyvinyl chloride (PVC) requires phthalates in the manufacturing process to form one of the most used plastic products (PVC along with polyethylene and polypropylene) (Plastics Europe, 2015). This would indicate that phthalates are the most significant plasticiser and although less high profile when compared to BPA, need careful consideration.

Phthalate esters are not tightly or covalently bound to the plastic polymer and therefore can leach from the plastic material into the environment (Beach et al., 2013). These leached

phthalates can result in human exposure by direct contact but ingestion is the most common route of contamination (Schettler, 2006).

The phthalate DEHP is a *substance of very high concern* (SVHC) due to its endocrine disrupting properties according to Registration, Evaluation, Authorisation and Restriction of Chemicals by the European Chemical Agency (REACH, 2016) (REF). This recognition of the dangers of DEHP led to it being banned in the EU as of 21 February 2015 (REACH, 2015). This ban is supported by research showing developmental perturbations of the testis due to exposure, at concentrations previously reported as having no effect, *in utero* using a rodent model (Jones et al., 2014).

1.11 CONTEMPORARY INDUSTRIAL CHEMICALS AND PBDE

High resistance to degradation is what makes synthetic organobromine compounds useful as industrial chemicals and flame retardants but this characteristic also makes these compounds persistent in the environment. PBDE is a commonly used flame retardant which is similar in form to Dioxins and PCBs and there is much concern over possible associations with human health problems and cancers (Solomon & Weiss, 2002; Birnbaum & Staskal, 2004).

Also in common with PCBs, PBDEs have 209 congener formulations with the number of bromine atoms determining the congener number. The lower brominated (five or fewer) PBDEs are of greater concern as their smaller size and molecular weight make them more detrimental to health due in part to the ease with which they enter the body. For this reason the use of PBDEs has been restricted since 2003 (Restriction of Hazardous Substances Directive) in Europe with only one of the form (Decabromodiphenyl ether) currently being allowed. The United Nations Environmental Programme, Stockholm Convention, has identified the PENTA and DECA formulations on its list to eliminate and/or restrict use (Stockholm Convention on POPs, 2009). Commonly used forms of PENTA and OCTA are of specific concern due to them being persistent (Environment Canada, 2004) and bioaccumulative (Ikonomou et al., 2002) with these two properties supported their ban. The ubiquity of PBDE has been demonstrated by detectable concentration concentrations found in remote artic locations distant from their site of production and use (Wang et al., 2005).

The Deca form of PBDE has been registered as a substance of concern in the Voluntary Children's Chemical Evaluation programme (VCCEP) operated through the Environmental Protection Agency (EPA). This programme evaluates in order to allow informed decisions on environmental chemicals with suspected ill health. Consequently it could be expected, that with this justification, a complete ban of all forms of PBDE.

Like most environmental pollutants, exposure to PBDE may be via ingestion, absorption or inhalation but worryingly a time of greatest exposure to PBDEs may be via breast milk when nursing (Hernik et al., 2013). The concentrations of PBDEs found in human tissue and serum is increasing year on year(Law et al., 2014). This phenomenon is demonstrated by concentrations of PBDE found to be occurring in breast milk from 1970 to 2000 (Solomon & Weiss, 2002b), as demonstrated in figure 1.3.

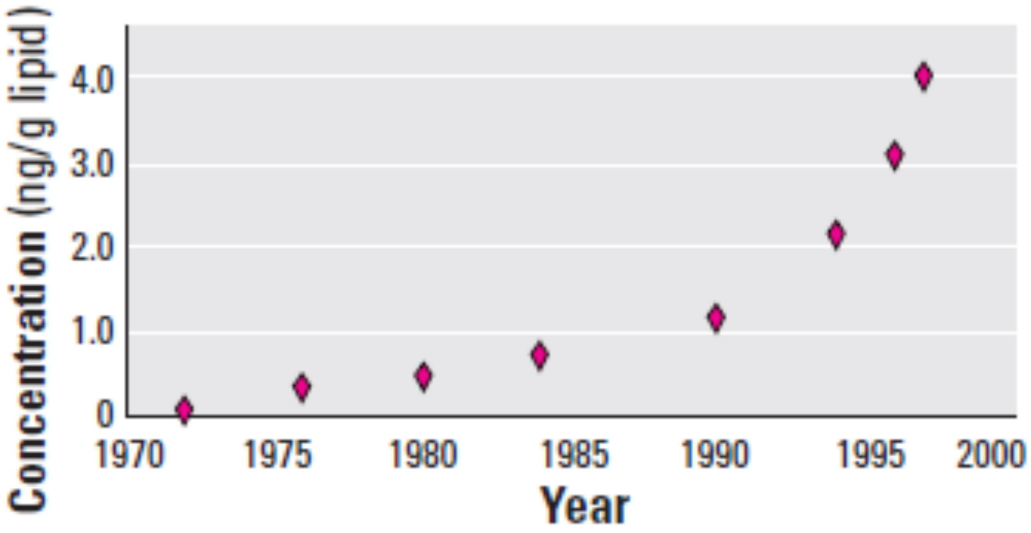


FIGURE 1-3 MEAN CONCENTRATIONS OF PBDE IN HUMAN BREAST MILK FROM ACHIEVED SWEDISH SAMPLES FROM 1972 – 1997 (SOLOMON & WEISS, 2002)

Exponential increases in PBDE concentrations have been observed in other species in parallel to that of human breast milk demonstrated in Figure 1.3. In seal blubber collected in northern Canada, concentrations of PBDEs increased in equilibrium with global production concentrations (Ikonomou et al., 2002) which are estimated to be 380 000 tonnes by 2020 (Abbasi et al., 2015).

PBDEs are one of the most prevalent EDs and are found in increasing concentrations in both animal and human systems. These chemicals are persistent in the environment and bioaccumulative in tissue. There have demonstrable effects on laboratory animals and suggested ill health implications in humans. Even amongst the group of compounds suggested as EDs, all these factors present PBDE as a chemical which presents cause for concern.

1.12 TISSUE CONCENTRATION CONCENTRATIONS OF PCB, DEHP AND PBDE

Concentrations of PCBs in tissue and serum of man and other species varies dependant on environment and trophic concentrations. As evidence of this, two top carnivores living in artic regions had measurable concentrations of 40 PCBs. This was shown in dogs fed a traditional seal blubber diet (2707±496 ng/g) and in Polar bears (4201±771ng/g) showing relatively high concentrations in adipose tissue (Verreault et al., 2008). Both of these species, high on trophic hierarchy, also showed relatively high concentrations in blood plasma. Indigenous and Inuit peoples living on a traditional diet have higher concentrations of PCBs with foetal and early life exposures at concentrations associated with ill health (Dallaire et al., 2014). Worryingly, the concentrations found when sampling the cord blood of new-borns was higher (132.2 μg/kg lipids) than that found in children (72.1 μg/kg lipid). Of equal concern was the concentrations of heavy metal (lead) followed the same pattern. These studies demonstrate the bioaccumulation, persistence and ubiquity of PCBs.

Concentrations of PCB found in a range of other non-polar and regional species and media are variable. The range, examples of which are shown in Table 1.3, is from below the limits of detection to 123.2 μg/kg lipid (Dallaire et al., 2014) which is lower than the relatively high concentrations reported in polar bears. Concentrations within this range are therefore relevant to the environment for this species in this region. This environmental relevance when considering concentrations is an important concept for experimental methodologies when working with EDs as a large variation across regions and species will be expected. This is exemplified in Table 1.3.

TABLE 1-3 CONCENTRATIONS OF ΣPCBS AND SPECIFIC CONGENER CONCENTRATIONS REPORTED IN SERUM AND TISSUE ACROSS A RANGE OF SPECIES

Congener(s)	Species	Medium	Concentration	Reference
Σ 52 101 138 153 180	Caprine	Seminal Plasma	1.44 ±0.94 ng/ml	(Kamarianos, et al, 2003)
Σ 52 101 138 153 180	Porcine	Seminal Plasma	0.64 ±0.09 ng/ml	(Kamarianos, et al, 2003)
Σ 52 101 138 153 180	Bovine	Seminal Plasma	1.06 ±0.29 ng/ml	(Kamarianos, et al, 2003)
Σ 52 101 138 153 180	Ovine	Seminal Plasma	1.21 ±0.64	(Kamarianos, et al, 2003)
Σ 20 52 60 77 101 105 118 138 153 156 180	Feline	Adipose tissue	1.99 (6.22–886.28) ng/g	(Storelli, et al 2009)
Σ 20 52 60 77 101 105 118 138 153 156 180	Canine	Adipose tissue	41.61 (4.07–258.66) ng/g	(Storelli, et al 2009)
Σ 74 99 118 138 146 153 156 170 180 183 187	Human	Serum	142 ± 92.2 ng/g	(Kalantzi, Geens, Covaci, & Siskos, 2011)
153	Human	Serum	88 (3–5500) ng/g	(Spanò et al., 2005)
153	Human	Serum - pregnant	21.16 (0.89 - 156.25) ng/g	(Jarrell, Chan, Hauser, & Hu, 2005)
153	Human	Breast milk	12.19 (2.69 - 38.24) ng/g	(Jarrell, Chan, Hauser, & Hu, 2005)
153	Human	Follicular fluid	0.15 ng/g	(De Felip, Domenico, Miniero, & Silvestroni, 2004)
153	Human	Serum	43.2 ± 27.1	(Kalantzi et al., 2011)
153	Human (neonate)	Cord blood	123.2 µg/kg lipid	(Dallaire et al., 2014)
153	Human (child)	Plasma	73.1 µg/kg lipid	(Dallaire et al., 2014)

Concentrations of PBDE have been reported in a variety of tissue and serum across a range of species. In human cord blood sampled from 108 Korean infants, the ΣPBDE (seven congeners) had a mean of 8.37 (± 6.381)ng/g lipid with a range of < LD to 29.407 ng/g (Kim et al., 2009). These concentrations were lower than those reported for pregnant women when blood serum concentrations were tested in 389 pregnant American women with concentrations being 19.1 ng/g lipid (Vuong et al., 2015). It is difficult to draw conclusions from the discrepancy in these reported concentrations, as there are other factors, such as geographic location, which must be considered. In a separate study, the PBDE congeners in three groups, children, parents of these children and older adults, showed a significant difference in concentrations of PBDE (Wu et al., 2006). Worryingly, children had the highest concentrations of all the groups. There was also a correlation between lifestyle factors, such as diet and household concentrations, and concentration of PBDEs. Also interesting to note is that PBDE 47 had a Frequency of Occurrence of 100% in the children's group. That is, all the subjects tested positive for concentrations above the limit of detection.

Concentrations of PBDE in tissue also showed variability. Fish from a lake in Central China had tissue concentrations of PBDE 47 of 58.47 ng/g(Yang, Fu, Teng, & Yang, 2015) and this was also the congener with the highest concentration. PBDE concentrations have been measured in the tissue of other species. For example, both Bald Eagles (*Haliaeetus leucocephalus*) and River Otters (*Lontra Canadensis*) were found to have accumulations of PBDE congeners in liver tissue (Dornbos et al., 2015). These tissues contained PBDE 47 at the highest concentration making up > 50% of the Σ_4 PBDE concentrations in both species. Eagles showed concentrations of 74.3 (±266.7) ng/g and the otter had a lower concentration of 16.3 ± 16.4 ng/g. The concentrations in the otter were stated as representative of other wildlife species but the eagle, with a lipid adjusted Σ_4 PBDE concentration of 5274.5 ± 19,896.1 ng/g was thought to be among the highest

concentrations found in birds worldwide. This further study also demonstrates the inter animal variation of EDs with a range in the eagle and otter respectively of <LD - 1,538.8 ng/g and 0.5 to 72.9 ng/g.

Human breast milk has also been shown to contain concentrations of PBDEs which has worrying implications for infants. When testing samples from 35 women living in Birmingham UK, all samples contained PBDE 47 with mean concentrations of 3.5 ng/g lipid (Abdallah & Harrad, 2014). This research also suggested that intake by UK adults via inhalation and ingestion resulted in somatic bioaccumulation with subsequently high concentrations of exposure to both the adults and breast feeding infants. The concentrations reported, using PBDE 209 as a reference congener, were comparable to those found in Australia with both countries having low concentrations of this congener compared to 18 other reports (Law et al., 2014). Some of the highest values for PBDEs are reported in Canada and the USA, specifically California which has historically strict fire retardant laws (PBDE is used as fire retardant) (Morgan & Gilman, 2013). Another study, which seems to confirm this, reported concentrations in Canadian women of PBDE 47 at 46.63 and Σ_7PBDE of 82.242 ng/kg lipid(Ryan & Rawn, 2014).

PBDE has also been detected in human semen samples (Liu et al., 2012). The highest concentrations were reported for PBDE 209 with PBDE 47 showing the second highest concentrations. Interestingly, PBDE was reported in all samples with PBDE 209 in only twelve of fourteen samples suggesting a higher ubiquity for PBDE 47. The mean level for all PBDE congeners was 13.2 ng/g lipid.

As a plasticizer, DEHP has become ubiquitous in the environment and is a known endocrine disruptor (Martinez-Arguelles et al.,. This chemical has an environmental ubiquity which has resulted in detectable concentrations of this pollutant in human and animal tissue and fluid media. Due to it not being as persistent and bio accumulative as other industrial chemicals,

DEHP is transient and more quickly metabolised. In spite of this, DEHP has been detected in 78 samples of breast milk from Bavarian women with a mean level of 3.9 ng/g (Fromme et al., 2011). Concentrations in breast milk in Korean women have been estimated to result in infant consumption concentrations ranging between 0.91 and 6.52 µg/kg body weight (bw)(Kim et al., 2015). As well as human breast milk, bovine milk sampled in Belgium contained relatively high concentrations of 463 µg/kg lipid (range: 312 - 535µg/kg lipid)(Fierens, Van Holderbeke, Willems, De Henauw, & Sioen, 2013).

Blood plasma when tested also proved to be contaminated with DEHP and worryingly when the cohort was divided into two groups, divided by age, the lower age group (16-39yo) reported significantly higher concentrations than did the higher age group (40 – 63yo) (Wan et al., 2013). Another study also detected DEHP in human blood plasma (Durmaz et al., 2010) with concentrations detected in healthy children (n = 21) reported as 3.09 (± 0.9) µg/ml blood plasma. Interestingly, and the focus of this research, the same study reported that children with gynecomastia had a significantly higher mean level of DEHP (4.66 ±1.59) and its metabolite MEHP (3.09± 0.90) µg/ml blood plasma than a control group of healthy children. Also of interest is that all samples (n = 61) contained detectable concentrations of DEHP meaning a frequency of occurrence of 100%. These studies suggest that there is an association between DEHP concentrations and pubertal gynecomastia which is likely the result of hormonal perturbations induced by elevated oestrogenic and/or anti-androgenic activity of the contaminant. The foetus and neonate are particularly sensitive to small endocrine changes and consequently, their exposure to these, and other, substances are associated with reduced adult fertility and reproductive health. This has been shown experimentally in laboratory rodents and sheep (Rhind et al., 2010; Sharpe & Skakkebaek, 2008). Therefore, the general concept that

anthropogenic substances are having an impact on normal endocrine function in humans and a range of animals worldwide is now undisputed.

The mechanisms by which DEHP, PCB and PBDE perturb reproductive development and ultimately fertility cannot be clearly stated but there has been some research suggesting direct actions. Many researchers generally suggest an endocrine action (Bay et al., 2006; Guzmán & Zambrano, 2007; Bellingham, Fiandanese, et al., 2012) without specifically identifying the point at which perturbations occur. One theory is that the exogenous substances have the ability to mimic the action of endogenous hormones. This direct action would be via the nuclear hormone receptor. There have been reports that PCBs may act direction on ER (Salama, et al., 2003) and additionally on both AR and ER (Bonefeld-Jørgensen et al., 2001). The ED, DEHP is also reported to act on the oestrogen system via ER binding (Ghisari & Bonefeld-Jorgensen, 2009) additionally, PBDE is a suspected oestrogen agonist acting via the ER (Kojima et al., 2009). This suggests that certain EDs can compete with endogenous ligand binding hormones resulting in a potential agonistic or antagonistic effect. This hormone receptor binding may also reduce hepatic clearances of endogenous hormones (Kabir et al., 2015) due to blocking naturally occurring hormones and therefor increasing circulating concentrations.

Endocrine disruptors may interact synergistically when combined in whole mixtures (Rhind et al., 2010; Rignell-Hydbom et al., 2007; Stavrakakis, 2008) or, alternatively it has been suggested they may act antagonistically (Kortenkamp, 2008). These two seemingly contradictory mechanisms of action demonstrate the unpredictability of mixtures of chemical pollutants.

1.13 THE IMPORTANCE OF ENVIRONMENTALLY RELEVANT CONCENTRATIONS

A majority of published experimental studies have used non-physiological (high relative to the environment) concentrations of single environmental chemicals. While these studies may illustrate an effect, the relevance to actual environmental exposure is debatable. EDs do not follow classical dose response curves and therefore traditional toxicological approaches to their function are not fully appropriate. These non-classical dose responses are described as Non Monotonic Dose Response Curve (NMDRCs) (Vandenberg et al., 2012). The range of possible dose response curves is outlined in Figure 1.4.

The dilemma of NMDR and low environmental concentrations is seen in the World Health Organisation guidelines to evaluate safe exposure concentrations for certain EDs using toxic equivalency factors (Van den Berg et al., 2006). The data used to create these recommendations are largely based on non-physiological experimental concentrations and the testing of single chemicals. Real world exposure is however characterised by exposure to mixtures of chemicals at low environmental concentrations. To address these issues, a sheep model of "real life exposure" to environmental chemicals has been established. Male lambs, from pregnant ewes exposed from mating to mid-gestation to EDs contained in processed human sewage sludge fertiliser, have fewer Sertoli and Leydig cells (Paul et al., 2005). In addition 2 chemical types, known to have endocrine disrupting activity (Diethylhexyl phthlate (DEHP); 2 PCB congeners (101, 118) are present at higher concentrations in the foetus than in the mother (Rhind et al., 2010).

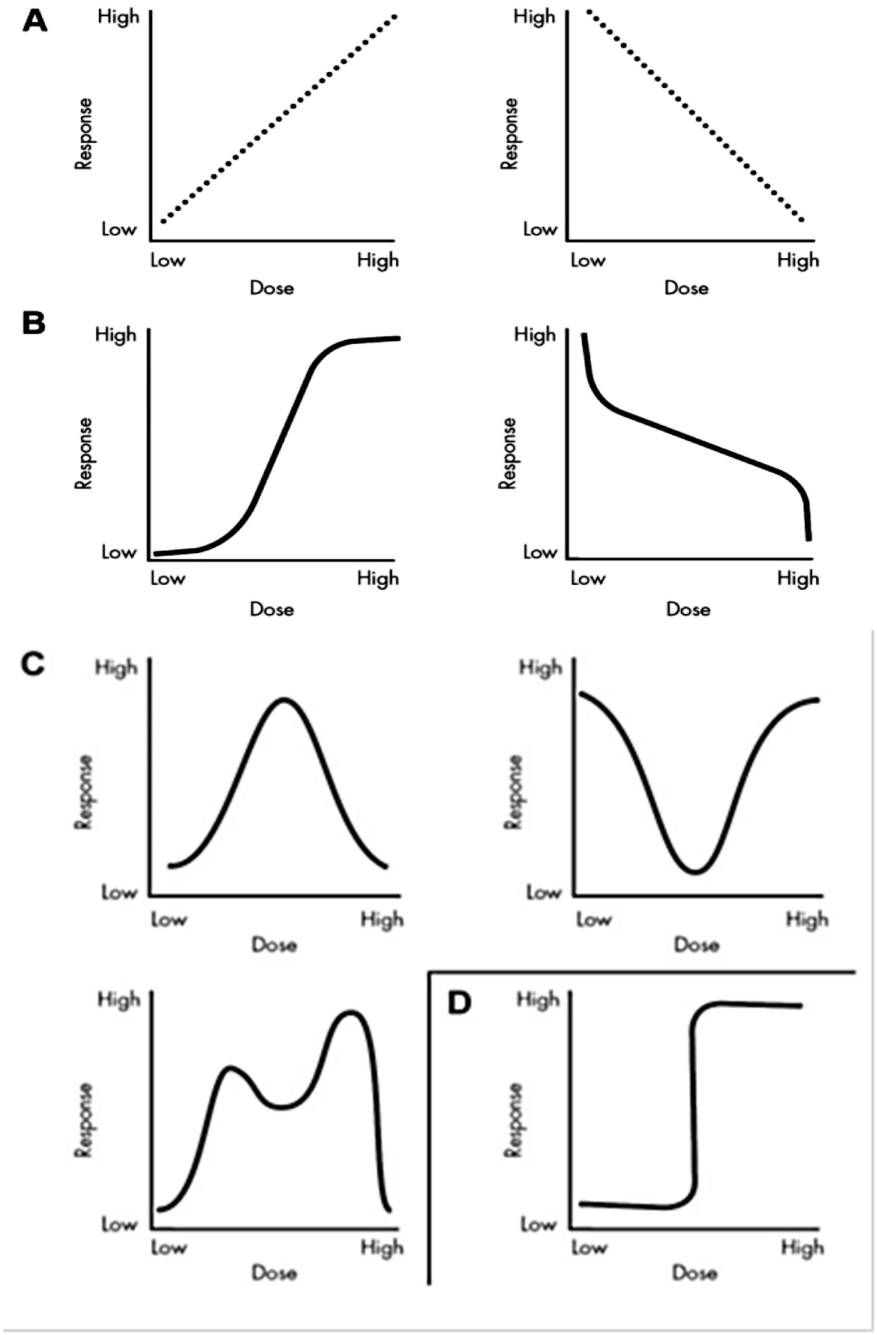


FIGURE 1-4 EXAMPLES OF DOSE-RESPONSE CURVES. A) LINEAR RESPONSES, B) EXAMPLES OF MONOTONIC, NONLINEAR RESPONSES. IN THESE EXAMPLES, THE SLOPE OF THE CURVE NEVER CHANGES SIGN, BUT IT DOES CHANGE IN VALUE. C) DISPLAYED ARE THREE DIFFERENT TYPES OF NMDRCS INCLUDING AN INVERTED U-SHAPED CURVE, A U-SHAPED CURVE, AND A MULTIPHASIC CURVE. ALL OF THESE ARE CONSIDERED NMDRCS BECAUSE THE SLOPE OF THE CURVE CHANGES SIGN ONE OR MORE TIMES. IT IS CLEAR FROM THESE CURVES THAT KNOWING THE EFFECT OF A DOSE, OR MULTIPLE DOSES, DOES NOT ALLOW FOR ASSUMPTIONS TO BE MADE ABOUT THE EFFECTS OF OTHER DOSES. D) A BINARY RESPONSE IS SHOWN, WHERE ONE RANGE OF DOSES HAS NO EFFECT, AND THEN A THRESHOLD IS MET, AND ALL HIGHER DOSES HAVE THE SAME EFFECT (VAN DEN BURG ET AL., 2012).

A second sheep model developed in Nottingham (Lea et al, unpublished) has exposed pregnant ewes specifically to DEHP and the two PCB congeners in the diet at concentrations equivalent to the sewage sludge model (i.e. environmental concentrations). Preliminary findings have shown that late gestation male foetuses exposed via the mother to these chemicals have altered testicular and pituitary development (Lea et al., unpublished; Lea, Byers, et al., 2014).

In the sewage sludge sheep model, there is emerging evidence of an adult male phenotype since exposure to ECs both in utero and post-natally to weaning, induced reproductive abnormalities in a subset of offspring. Bellingham et al (2012) reported that 5 of 12 male offsprine exhibited major spermatogenic and testicular abnormalities (Bellingham, McKinnell, et al., 2012).

1.14 Sentinel Species for the study of Endocrine Disruption and Reproductive Health

To fully explore the complex and multifaceted puzzle of endocrine disruption and reproductive health, experimental models only provide part of the solution. While the sheep has proved an excellent model of real-life exposure, my work will focus on the dog as a 'sentinel' species for human exposure to EDs. Not only does the dog more closely share our environment than any other species, but it also serves as an ideal model for exposure of human infants and children to EDs. It has been suggested that their close proximity to flooring and household fabrics (Schmidt, 2009) and their mouthing of toys and other objects (Enriquez et al., 2009; Wooten & Smith, 2013) may equate to similar exposure concentrations to those of children. There have

been many other animals proposed as experimental model and Table 1.4 lists a more full range of animals proposed as sentinels.

Definitions of sentinel have been reviewed by (O'Brien et al., 1993) who concluded that: "Sentinels allow graded evaluation and quantification with specific and exclusive reference to implication for human health, and give early warning of those implications". The term biomonitor has also been used (López-Alonso et al., 2007) and could further refine the definition. The dog would seem to meet these requirements

TABLE 1.4 ANIMAL MODELS PROPOSED AS SENTINELS FOR THE STUDY OF ENVIRONMENTAL EXPOSURE TO EDS AND HUMAN REPRODUCTIVE HEALTH / DEVELOPMENT.

Proposed Model	Specific area of study	Evidence	Reference
Companion Animals			
Feline	Low level chronic exposure to PBDE	Empirical	(Dye et al., 2007)
Canine	Metal concentration in tissue.	Empirical	(López-Alonso et al., 2007)
Canine	PCVs	Empirical	(Schilling, Steele, Harris, Donahue, & Ing, 1988)
Canine/Feline	TCDD	Empirical	(Schilling & Stehr-Green, 1987)
Production Animals			
Bovine	Sperm quality	Empirical	(Wahl & Reif, 2009)
Bovine	Tissue and fluid concentrations of PCBs, OCPs, PBDEs	Empirical	(Petro et al., 2010)
Wildlife			
Primate	Germ cell differentiation/Testicular germ cell tumor	Empirical	(Mitchell et al., 2008)
Mink	Hg, PCB	Review	(Basu et al., 2007)
Marine Mammals	Anthropogenic toxins	Case Study	(Bossart, 2006)(Jepson et al., 2016)
General			
Domestic Animals	EDs	Review	(Majdič, 2010)
Mammals	Toxic environmental contaminants	Review	(O'Brien et al., 1993)
Animals	Environmental Chemicals	Review	(van der Schalie et al., 1999)
Companion Animals	Public health / environmental contaminants	Review	(Schmidt, 2009)

So, as a mammal having similar physiology to humans and closely sharing our environment, the dog could be proposed as a sentinel or biomonitor. This model could provide quantifiable reference data which will give insight into the implications for human health of deleterious environmental substances like EDs.

1.15 HYPOTHESIS AND AIMS

My hypothesis is that as a companion animal sharing its environment with humans, the dog is exposed to the same Endocrine Disruptors (EDs) as humans and therefore may show negative temporal trends in fertility and perturbations in reproductive health. Therefore the aim of the projects is to critically examine the relationship between reproductive health and fertility and environmental endocrine disrupting chemicals using a canine research model. The following objectives are designed to achieve this aim.

1. Identify trends in canine reproductive health, specifically those related to human TDS.
2. Determine the presence and concentrations of EDs in canine reproductive tissues and semen.
3. Determine the concentrations of target EDs across a range of geographic areas in both the UK and Scandinavia.
4. Carry out histological analysis of testis tissue from different geographic areas and examine these results against other suggested causative factors related to fertility and reproductive health.
5. Measure the effects of short term acute exposure on canine spermatozoa and testes tissue exposed to certain EDs.
6. Examining the mechanisms by which certain EDs perturb testis function particular endocrine function

These studies will ultimately enable further evaluation of the dog as a sentinel model for the examination of reproductive health and fertility in humans.

Chapter 2 MATERIALS AND METHODS

All sperm and testis collections and measurements were performed in accordance with relevant guidelines and regulations and all experimental protocols were approved by the University of Nottingham School of Veterinary Medicine, Ethical Review Committee. All samples were collected as part of routine breeding management. Sperm was collected as part of routine reproductive examination of stud dogs and residual canine testis was collected from normal healthy dogs during routine castrations and subject to full owner consent.

2.1 Identification of Temporal Trends in Canine Fertility and Reproductive Health

Longitudinal data on sperm quality of a cohort of dogs kept as part of a national service dog breeding programme was provided for identification of temporal trends. Records of semen analysis collected annually (1998 – 2014) from stud dogs used in an assistance dog breeding programme provided the raw data. The dogs were of five breed types; Labrador retriever, golden retriever, curly coat retriever, collie and German shepherd. The mean number of dogs in the programme was different each year and ranged between 42 – 97 which resulted in a total of 1925 ejaculates from 232 different dogs. As a means of quality control, all semen was collected and evaluated by a single experienced person or one of three technicians trained by him.

In 1998 it was clear that the PNM for the population was significantly lower than it had been in the majority of the 10 preceding years. Therefore in years 1999 to 2000, dogs with poor semen quality (dogs with any of the following measurements: <60% normal

forward progressive motility, <60% morphologically normal sperm, <150 million sperm) were removed from the programme. A management practice to eliminate, from the breeding programme, the dogs with the lowest fertility measures (PNM; total sperm output (TSO)) was achieved during 1999 and 2000. This resulted in an overall improvement in the semen quality of dogs in the programme. On the basis of this intervention, trends in semen quality were evaluated and data are presented as two separate periods: 1988-1998 and 2001-2014 inclusive.

The same population of dogs from the National Breeding Centre (GDBA) provided data on pups identified with cryptorchidism between 1995 – 2014. Specifics of the dog group described previously. The number of cases of cryptorchidism was calculated showing an incidence rate per 100 dogs. This is based on the number of animals identified in the data base as cryptorchid and the number of male pups born that year.

2.2 CANINE TESTES TISSUE COLLECTION

All dog owners providing tissue samples completed an informed consent declaration which can be seen in Appendix A. A questionnaire was created to record information on dogs presented as potential tissue donors which can be seen in full in Appendix B. This contained questions on the general background of the dogs, dietary information, clinical history, additional health and lifestyle factors as well as details of the castration procedures. Once this was completed the dogs were deemed eligible for tissue donation.

Testes samples were residual, collected during routine castrations from all dogs as well as opportunistic without an attempt to target specific breeds or types of dogs. Each testis was sequentially allocated a unique identification number which was marked on sample pots and all related data sheets. The attending veterinary surgeons were instructed to weigh the testis, sans epididymis, after castration and record weights for left and right testis on tissue data sheets. A 5mm thick disk was cut from the centre (coronal plane) of both the right and left testis. This disk was placed in Bouin's fixative for 6 hours along with the epididymis. After 6 hours fixation the tissue was transferred to a 70% ethanol solution for storage at room temperature. A small sample, approximately 4mm^3, was cut or scraped from the peripheral edge of one of the remaining tissue section. This was collected on the peripheral edge so as to avoid collecting only rete testis. This sample was submerged in 'Qiagen Allprotect Reagent' for nucleic acid preservation and stored at 4°C. The remainder of the tissue from both testes was wrapped in aluminium foil (so as to avoid contamination, especially from plastic) placed in a paper wax lined padded envelope, and stored at -20°C for future chemical analysis.

Total residual canine testis tissue was collected during routine castrations in the UK through the GDBA and participating veterinary practices (n = 36), Finland through the Finnish Guide

Dogs Association (n=10) and Denmark (n = 19) through the Department of Large Animal Science, University of Copenhagen (and associated veterinary practices). Tissue samples were sent to the James Hutton Research Centre for chemical testing to determine background concentrations of PCB (congeners 28, 47, 101, 118. 138, 153, 180) PBDE (congeners 28, 47 99, 100, 153, 154, 183) and DEHP.

2.3 CHEMICAL TESTING OF COMMERCIAL DOG FOOD AND TESTES TISSUE AND OTHER MEDIA

As the testing was for ubiquitous environmental chemicals (and chemicals found in plastic), it was necessary that this testing was done by an ISO accredited laboratory (The James Hutton Research Station) to prevent sample contamination.

A range of commercially available dog food (n = 28) was tested for three representative EDs: Polychlorinated biphenyls (PCB), Polybrominated Diphenyl Ethers (PBDE) and Di(2-ethylhexyl)phthalate (DEHP). Fourteen samples of dry dog food and 14 samples of canned food were tested for PCBs (congeners 28, 47, 101, 118, 138, 153, 180), PBDEs (congeners 28, 47, 99, 100, 153, 154, 183) and DEHP (see Table 2.1 for chemical details and CAS numbers).

Canine testes tissue collected from the UK (n = 34), Denmark (n = 20) Finland (n = 10) were collected for testing of representative EDs as described above. Additionally, bitches milk (n = 11), prostatic fluid (n = 13), and fraction 2 ejaculate samples (n = 3pooled samples: 2 pools of 5 and one of 4 ejaculates) were collected for testing for concentrations of the same range of chemicals.

The analytical method used to determine concentrations are described elsewhere (Rhind, et al., 2005; Rhind et al., 2010). Briefly, internal standards were added to 1 g tissue samples followed by extraction with dichloromethane : isohexane (v/v 1 : 1) at 55 C for two hours. The extract was filtered and evaporated under nitrogen. The sample clean-up was achieved by absorption chromatography (10 g acid modified silica column which was conditioned by 40 ml isohexane). The eluate, solution extracted by solvent (100 ml isohexane), was collected and concentrated by rotary-evaporation and gentle nitrogen flow and transferred to GC vials, ready for further analysis.

DEHP: samples (approximately 1 g dry weight) were spiked with 4 mg internal standards of d4-diethylhexyl phthalate (DEHP) and then extracted by Dionex ASE 200. (Solvents: dichloromethane. Temperatures: 100°C. Pressure: 1500 psi. Static time duration: 5 minutes. Flush volume 60% and purge 60 seconds). The extracts were dried down under a gentle stream of nitrogen and then re-dissolved in 0.5 ml isohexane. Sample clean- up was achieved using solid phase extraction (SPE) Strata NH2 columns (1 g per 6 ml) pre-washed by diethyl ether. The column was conditioned with 10 ml of isohexane and the samples were loaded and then removed with solvent, 12 ml isohexane, and followed by 16 ml 10% diethyl ether in isohexane for collection. The collected fraction was condensed to a small volume under nitrogen gas and then transferred to clear GC vials for further analysis. The samples were filtered and evaporated to dryness under nitrogen and re-dissolved in isohexane. Sample clean-up was conducted according to the protocols described above.

Five testes tissue samples were initially tested for PCB 153 and DEHP (mean 0.90 µg/kg; mean 0.31 90 µg/kg respectively). The mean concentrations of this initial cohort of testes was selected as being representative of canine testis concentrations and thus environmentally relevant. Consequently, these concentrations were used for the in vitro culture of tissue and sperm.

TABLE 2-1 CHEMICALS CHOSEN FOR TESTING WITH CONGENER CAS NUMBERS AND SYNONYMS.

Chemical	Congener	CAS number	Alternate name
PCB	28	7012-37-5	2,4,4'-Trichlorobiphenyl
PCB	47	2437-79-8	2,2',4,4'-Tetrachlorobiphenyl
PCB	101	37680-73-2	2,2',4,5,5'-Pentachlorobiphenyl
PCB	118	31508-00-6	2,3',4,4',5-Pentachlorobiphenyl
PCB	138	35065-28-2	2,2',3,4,4',5'-Hexachlorobiphenyl
PCB	153	35065-27-1	2,2',4,4',5,5'-Hexachlorobiphenyl
PCB	180	35065-29-3	2,2',3,4,4',5,5'-Heptachlorobiphenyl
PBDE	28	41318-75-6	2,4,4'-Tribromodiphenyl Ether
PBDE	47	5436-43-1	2,2',4,4'-Tetrabromodiphenyl Ether
PBDE	99	60348-60-9	2,2',4,4',5-Pentabromodiphenyl Ether
PBDE	100	189084-64-8	2,2',4,4',6-Pentabromodiphenyl Ether
PBDE	153	68631-49-2	2,2',4,4',5,5'-Hexabromodiphenyl Ether
PBDE	154	207122-15-4	2,2',4,4',5,6'-Hexabromodiphenyl Ether
PBDE	183	207122-16-5	2,2',3,4,4',5',6-Heptabromodiphenyl Ether
DEHP	n/a	117-81-7	Dioctyl phthalate; Di(2-ethylhexyl)phthalate

2.4 Sperm Culture Methodology

Ejaculates were collected from stud dogs (n = 7) located at the Guide Dogs for the Blind National Breeding Centre (Leamington Spa). All collections and laboratory analyses followed accepted GDBA protocols. The sample group consisted of healthy dogs of three breeds (German Shepherd (n=1), Labrador (n=4), Golden retriever (n=2)) aged between 2 – 8 years. An abstinence period of a minimum of one week was observed.

Collections were performed by digital manipulation of the penis as described by Seager and Fletcher (1972) and Kutzler (2005). Briefly, the procedure was performed in front of an oestrous bitch, especially with a naive dog. If an oestrous bitch is not available, then a swab from an oestrous bitch was used. Massage of the dog's penis through the prepuce was done to allow the dog to present with a partial erection. The dog's penis was held in the left hand and the collection container with the right, direct the penis into the collection cone. Firm pressure applied between the index finger and the thumb directly behind the bulbus glandis is required.

The ejaculate is released in three fractions: presperm fraction, sperm rich fraction and prostatic fluid. These were differentiated visually as the presperm and prostatic fractions are clear with the sperm rich fraction being cloudy. The second fraction only was collected.

Motility was assessed as percentage of normal forward progressive motility (PMN), morphologically normal live spermatozoa (Percentage Normal Sperm:PNS) and the total spermatozoal output (TSO) was measured using standard techniques (WHO, 2010).

All the ejaculates were assessed upon collection and found to be within normal ranges for pH, volume, odour and colour. A breeding technician recorded volume (ml), motility, sperm concentration, total sperm output, total number live sperm as part of a routine fertility

assessment done for the health screening of the breeding prior to use for and peripheral to the research experiment. Slide smears were prepared for morphological assessment using Nigrosin/Eosin Stain according to WHO (2010). Briefly, a stain of equal part Nigrosin (10%) and Eosin (5%) was prepared. A sample of sperm rich fraction of the collected ejaculate was incubated for a minimum of 2 minutes in the stain (1:4 ratio sperm to ejaculate). A histological smear was created on a standard microscope slide and allowed to dry. These slides were retained for future analysis.

The sperm was extended (20:1) in a Phosphate Buffered Saline (PBS) ethanol solution (0.1% solvent as described by Pocar, 2005) containing 4 concentration of three individual EDs and one mix of EDs (PBDE 47, PCB 153, DEHP, mix PCB 153/DEHP) dissolved in ethanol (0.1% in final solution)[see chapter 3 for rationale behind EC selection]The control solution contained PBS and 0.1% ethanol only. The concentrations of PCB 153 and DEHP incubated with sperm were 2X, 10X and 100X the mean background concentrations found in testes identified in chapter 3 (Mean PCB 153 = 0.90 µg/kg; DEHP = 0.31 90 µg/kg). The sperm rich fraction of the ejaculate was cultured in concentrations of PBDE 47 at 2X and 10x (100X concentration was not possible with PBDE 47 for methodological reasons) the mean background concentrations found in testes is described in chapter 3 (Mean PBDE 47 = 0.79; PCB 153 = 0.90 µg/kg; DEHP = 0.31 µg/kg)

Samples were collected from each dog daily for three days. These collections were treated with chemical concentrations as described above on days 1 (100x),2 (10x) and 3 (2X) respectively. Control exposures were done for each collection on each day.

To assess the possible confounding effect of the solvent, a 0.1% ethanol / PBS solution was tested against a control solution containing no ethanol on each of the motility parameters.

2.5 COMPUTER ASSISTED SPERM ANALYSIS (CASA)

Mixed semen (10µl) was placed via micropipette on a Helber counting chamber slide with a depth of 0.02mm (Hawksley, UK), covered by a 22x22mm coverslip and assessed for motility using a Hobson Sperm Tracker (Hobson Tracking Systems Ltd, UK), as described by Smith (Smith, 2000), using settings described by Hobsons Tracking Systems Ltd. Each treatment and the control were tracked for one minute at four time points (0, 60, 120, 240 minutes).

All parameters of sperm motility, as defined by (Holt, O'Brien, & Abaigar, 2007), were measured using the sperm tracker (See Table 2.2). These were straight line velocity (VSL), curvilinear velocity (VCL), average path velocity (VAP), straightness (SLR), linearity (LIN), amplitude of lateral head displacement (ALH) and beat cross frequency (BCF). The Hobson's Sperm Tracker also recorded dance mean (DMN), pause-variation of VSL (PFT), mean angular head displacement (MAD), and progressive motility (WHO, 1996). Mean angular displacement (MAD) is defined by WHO (2010) as: the time-average absolute values of the instantaneous turning angle of the sperm head along its curvilinear trajectory. The measure of DMN is defined as mean ALH/LIN/100 (Robertson, 1988) and PFT is the pause or variation of VSL (ug/kg) which is a measurement of how much a spermatozoa changes its movement along a track (Hobson Sperm Tracker (users manual)). Measurements of percent active, percent hyperactive and percent motile as defined by WHO (WHO, 1987) were also obtained.

TABLE 2.2 DEFINITIONS OF SPERM MOTILITY DESCRIPTORS (FROM HOLT ET AL., (2007))

Descriptors	Abbreviation	Unit	Description
Curvilinear velocity	VCL	$\mu m\ s^{-1}$	Velocity of progression along the entire trajectory
Average path velocity	VAP	$\mu m\ s^{-1}$	Velocity of progression along the smoothed trajectory
Straight line velocity	VSL	$\mu m\ s^{-1}$	Velocity of progression from first to last coordinates
Beat cross frequency	BCF	Hz	Frequency that the sperm head crosses the smoothed trajectory
Amplitude of lateral head displacement	ALH	μm	Mean lateral sperm head displacement along the smoothed trajectory
Linearity of track	LIN	%	VSL/VCL × 100
Straightness of track	STR	%	VSL/VAP × 100

The above methodology was repeated for each ED (PBDE 47, PCB 153, DEHP and mix PCB153/DEHP) at each concentration (2x, 10x, 100x) to provide 0 minute results for the four chemicals and the control solution and then repeated in its entirety again at 60, 120, 240 minutes. Semen was stored in a water bath at 37 C° during this time.

2.6 HYPO-OSMOTIC SWELLING TEST

The methodology for HOS has been described previously (England & Plummer, 1993) but briefly; a hypotonic solution (50 mOsm/l) using fructose (Sigma, UK), sodium citrate (Sigma, UK) and reverse osmosis water was prepared. Mixed semen (100µl) and control solution was added to 200µl HOS solution and incubated at 37^{o}C for 30 minutes. After inversion, 10ul was pipetted onto a glass slide and 200 sperm were observed (40X magnification). The number of sperm with and without swelling (curled tails), were counted, the latter being indicative of abnormal membrane integrity.

2.7 IDENTIFICATION OF GEOGRAPHIC LOCATION AND LAND USE CATEGORIES FOR TISSUE SAMPLES

The tissue samples were identified initially, for the purposes of identifying geographic variation, by national boundaries. The UK samples were additionally identified by residential post code data by intra national geographic boundaries, more precisely as either from the East Midlands, West Midland or Southeast.

A further categorisation based on post code location of all tissue was used to identify land use type for the residential location of the dogs. A geographic information system (GIS) utilizing Google Earth (Google Inc.) and CLC2006 was used to identify the specific location of the individual dogs residential post code. The CORINE Land Cover (CLC) is based on satellite images providing standardised and comparable data for the European geographic region. CORINE raster data 2006 version 17 (12/2013) Corine Land Cover (CLC), from European Environmental Agency (EEA) and Joint Research Centre of the European Commission was used to identify the land use type for each specific post code location of the home of the dogs from which tissue was collected.

More specifically, the map was overlaid with postcode points of dogs home locations identified through tissue data sheets. These data points were subsequently used to identify the corresponding CORINE category 1 land use. The postcode locations would therefore be identified as either: Artificial Surface, Agricultural Areas, Forest and Semi-natural Forest, Wetlands, Water Bodies. Due to reduced sample number only two categories, Agricultural and Artificial, were used for statistical analysis. The Artificial land use category is made up of urban fabric, industrial commercial and transport units, mine, dump and construction sites as well as artificial non agriculture vegetated areas. The Artificial land use category is made up of arable land, permanent crops, pastures and heterogeneous agriculture areas.

Tissue samples were collected and categorised as from three national regions. These regions included the countries of UK (n = 32), Finland: Helsinki area, (n = 12) and Denmark: Copenhagen area (n = 10). The tissue collected from the UK was divided into three groups representing the geographic regions of East Midlands (n = 7), West Midlands (n = 26) and Southeast (n = 15).

2.8 HISTOLOGICAL ASSESSMENT

All tissue samples were prepared for histological assessment and initial H&E staining. Upon arrival at the University laboratory, the tissue was rinsed in an ethanol solution (70%). If required the tissue was trimmed prior to processing. Tissue was place into individual histology cassettes and processed using a Leica TP1020 Semi-enclosed Benchtop Tissue Processor using a standard 18 hour programme. After processing the tissue was imbedded in paraffin wax for sectioning (5µm) on a rotary microtome (Leica RN2265). Tissue sections were place in a water bath and floated on to microscope slides subbed with poly l lysine (Polysine, Thermo Scientific, Hemel Hempstead) and placed on a heat plate (40°C). Slides were then place in an incubator (35°C) overnight.

Tissue samples were stained for initial histological analysis with Harris Haematoxylin and Eosin (H&E) using a standard protocol. Briefly, dewaxing was achieve in Histo-clear (National Diagnostic, UK) (2 X 5 minutes) then the tissue was serially rehydrated in ethanol solutions (100%, 90%, 70% X 2 minutes). Nucleus staining was achieved with Haematoxylin stain (5 minutes) prior to rinsing in running water. Staining was differentiated in acid alcohol solution (3% hydrochloric) and then rinsed in running water. Counter stain of Eosin was applied for 2 minutes prior to rinsing in water. Tissue was dehydrated in serial concentrations of ethanol (70%, 90% 100% X 2 minutes) prior to immersing for 5 minutes in Histo-clear. Prior to mounting with DPX, slides were place in Xylene for 5 minutes.

2.8.1 GROSS HISTOMORPHOLOGY

An initial scoring of the tissue samples was conducted to assess gross histomorphological structures. One tissue section from the median plane, (5 µm), stained with H&E, were examined under bright field microscopy at 40 and 200x magnification. One tissue section per testes was scored on four important components of testes anatomical structures: organisation of tubules, lumen structure, mediastinum/rete testes, capsule. Each of these was scored individually according to the criteria in table 2-3. This semi quantitative assessment was based on the quality of the tissue in comparison to typical healthy mature testes. Score is a maximum of ten with individual components weighting of 4, 2, 2, 2 respectively as describe in table 2.3.

TABLE 2-3 SCORING CRITERIA FOR GROSS HISTOMORPHOLOGICAL ANALYSIS OF TESTES TISSUE. CELLS CONTAINING NO TEXT INDICATE LIMIT BEYOND SCALE OF SCORE WEIGHTING IN THE RESPECTIVE CRITERIA AND IDENTIFIED WITH - .

Score	Organisation of tubules	Lumen structure	Mediastinum/rete testes	Capsule
0	Pathological, atypical organisation.	No obvious lumen in tubules.	Pathological, atypical.	Pathological, atypical.
1	0 – 49% of testes poorly organised.	Some areas with tubules absent.	Some areas of pathological or atypical mediastinum and or rete testes.	Thin in areas, poorly stratified.
2	Equal areas of disorganisation and typical organisation.	Typical luminal structures.	Almost wholly Typical mediastinum and rete testes structures.	Typical capsular structures
3	Largely organised as typical but still few areas (< 10%) disorganised.	*Limit beyond scale*	*Limit beyond scale*	*Limit beyond scale*
4	Typical, no areas of disorganisation.	*Limit beyond scale*	*Limit beyond scale*	*Limit beyond scale*

2.8.2 JOHNSEN SCORING

Further detailed histological analysis was completed using the method first described by Johnsen (1970) and modified for canine testes by Peters et al. (2000). Viewing at a magnification of 400x, H&E stained sections (one section per testis) were viewed under standard brightfield microscopy. The sample was traversed horizontally from the top left aspect of the section, moving across, down and back until 100 seminiferous tubules were assessed and scored according to the criteria outlined in Table 2.4. Tubules nearest to the capsule and rete testes were not assessed. The score applied to each tubule is based on the criteria outlined in Table 2.4 and the mean of the 100 tubules was attributed to the individual sample of tissue.

TABLE 2.4 CRITERIA FOR TESTES SCORING BASED ON ORIGINAL WORK OF JOHNSEN (1970) AND CANINE SPECIFIC MODIFICATIONS OF PETERS ET AL. (2000).

Score	Johnsen Scoring Criteria
10	Complete spermatogenesis with many spermatozoa. Germinal epithelium organized in a regular thickness leaving an open lumen, or stage 5 of the seminiferous cycle, with sufficient round spermatids.
9	Many spermatozoa present but germinal epithelium disorganized, with marked sloughing or obliteration of the lumen.
8	Only few spermatozoa (< 5–10) present in a tubular cross-section.
7	No spermatozoa but many spermatids present.
6	No spermatozoa and only few spermatids (< 5–10) present.
5	No spermatozoa, no spermatids but several or many spermatocytes present.
4	Only few spermatocytes (< 5) and no spermatids or spermatozoa present.
3	Spermatogonia are the only germ cells present.
2	No germ cells but only Sertoli cells present.
1	No cells in tubular cross-section.

2.8.3 TUBULE SIZE

Tubular size (diameter through the centre of the tubule lumen), as another quantification for analysis, was measured for 15 tubules selected randomly per testis tissue sample. This measurement was achieved using an imaging application (ISCapture 4.1.3 (2015)) integrated with a digital camera (Tucsen H series) and a standard laboratory brightfield microscope (CN-B10, Carton Optical). The mean size of the fifteen tubules was attributed to the individual testis tissue sample for statistical analysis.

2.9 IMMUNOHISTOCHEMICAL ASSESSMENT

Samples from the UK Finland and Denmark were processed for immunohistochemistry to stain CYP11A1 as a Leydig cell marker and indicator of steroidogenesis. Tissue sections (5 µm) on polylysine coated glass slides (Polysine©, Thermo Scientific) were dewaxed in histoclear and rehydrated serially though concentrations of ethanol (100, 90, 70 % x 5 minutes), RO water (5 minutes) and finally PBS buffer (2 x 5 minutes).

An antigen retrieval step was used to break any methylene bridges formed during fixation and to expose antigenic sites. This was achieved using a heat based method (as opposed to enzyme method). Slides were microwaved (4 X 5 minutes; 1 minute intermittent rest) in an citrate buffer (pH = 6.0). After heating, the slides were rested in same citrate buffer (20 minutes at ambient temperature)*.

Immunohistochemistry was carried out using the Vector ABC kit (Vector Laboratories, Burlingame, CA) following established protocols but briefly outlined below.

To avoid detection of non-specific enzyme in the tissue, an endogenous peroxidase block (3% H_20_2 x 5 minutes) was applied*. Slides were then transferred to an immunohistochemistry humidity chamber. An additional blocking solution (1% normal horse serum) was applied (20 minutes) followed by an additional avidin/biotin block solution (Lab Vision ™ – ultra V block, Thermo Scientific) and incubated for 5 minute at ambient temperature*.

Primary antibody (Anti-Cytochrome p450 (scc) (CYP11A1), Millipore, Merck Chemicals Ltd, Nottingham, UK) from a rabbit host (species reactivity: rat) was applied at a concentration of 0.1%. A rabbit IgG (Sigma, Poole, Dorset) solution (0.1%) was applied to negative controls.

Samples were cover-slipped and incubated in a standard laboratory fridge (4-5°C) within the IHC humidity chamber for 12 – 14 hours (overnight).

*A buffer wash (PBS: 1 x 5 minutes) was used between antigen retrieval, endogenous peroxidase block and primary antibody incubation steps.

After incubation with primary antibody, coverslips were removed in Phosphate Buffered Saline with Tween® 20 PBST solution (PBS + Triton X-100). A wash solution (PBST) was applied (2 X 5 minute) prior to secondary antibody* incubation (30 minutes). A further PBST was (2 x 5 minutes) was applied prior to incubation (30 minutes) in Avidin/biotynlated enzyme complex (ABC)* solution.

Staining was achieved by using a DAB substrate for immunoreactivity and a background counter stain of Haematoxylin was used (30 seconds). Sections were serially dehydrated (70, 95, 100% ethanol x 2 minutes) prior to mounting with DPX (Xylene prior to mounting x 5 minutes).

Immunohistochemically stained slides were visualised by standard brightfield microscopy (DMB500B, Leica, Germany). Images were captured (40x / sample) at 200x total magnification by sequential moving down and over, capturing 10 fields of view starting from each of the four "poles" of the sample. Per cent area stained was determined using image analysis software (Image Pro Premier version 9.1 software, Media Cybernetics, Inc, Rockville, MD). Through application of colour filtration the area of brown stained (DAB Substrate) as well as the area of background counter stain (haemotoxylin) was determined. The area of brown stain divided by the area of background stain produced a per cent area stained for DAB.

2.10 TISSUE EXPLANT CULTURE SYSTEM

2.10.1 TESTES EXPLANT CULTURE: OPTIMIZATION OF PROTOCOL

Explant culture protocols were adapted from Fowler et al. (2007) as well as Kasimanickam et al. (2013). Canine testes were collected from a veterinary hospital (Pride Veterinary Centre, Derby) associated with the University of Nottingham School of Veterinary Medicine and Science. Residual testes from routine castrations of post pubertal, otherwise healthy animals was transported in a thermal flask in pre warmed (37°C) transport medium (DMEM, 10% bovine foetal serum). Details of components of transport, sectioning and culture media found in Table 2.5

TABLE 2.5 DETAILS OF TRANSPORT, SECTIONING AND CULTURING MEDIA FOR TESTIS EXPLANT CULTURE.

Transport Medium (Serum Free)
Dulbeco's Modifided Eagle Medium(DMEM) – high fructose (500ml) ≈100 µg/ml Penstrep ≈50 µg/ml Gentamycin
Sectioning Medium (Serum Free)
Dulbeco's Modifided Eagle Medium/F12 (DMEM) – high fructose (500ml) ≈20 µg/ml Penstrep ≈20 µg/ml Gentamicin
Culturing medium
Dulbeco's Modifided Eagle Medium/F12 (DMEM) – high fructose (500ml) ≈10% Bovine Foetal Serum (50ml) ≈20 µg/ml Penstrep ≈20 µg/ml Gentamicin L-Glutamine 200mM (5ml aliquot)

The testes dissections were carried out in a sterile fume hood using established aseptic techniques. The Tunica vaginalis (parietal layer) and epididymis were removed from the testes and a 2.5 - 3mm disk (cross section) dissected from the centre of the testis with the tissue

submerged in sectioning medium throughout. Serial pieces of parenchyma, avoiding the central area containing the rete testis were removed using a 3mm biopsy punch.

Testes tissue samples were cultured in a 24 well culture plate with three biopsy sections per well in 1ml sectioning medium. These samples were placed in an incubator (Hera Cell 150i, Thermo Scientific) set at 37°C, 5%CO_2. During incubation the plates were placed on a shaker (Sea Star digital shaker) and subjected to a 30 minute wash period whilst agitating at 500rpm.

After the wash period, the tissue samples were transferred to a 24 well culture plate with each well containing 1ml culture medium. This culture plate was placed in a 37°c, 5%CO2 incubator resting on a plate shaker at 500rpm. Cultures of the tissue were carried out for four time periods for histological assessment of viability during culturing. Tissue was removed from culturing at 6, 12, 24 and 48 hours and fixed in Bouin's fixative for six hours..

Once removed from incubator, cultured tissue was prepared for microscopy by processing, sectioning, mounting and H&E staining as described above. The samples were examined under brightfield microscopy to assess viability at the four time points (6, 12, 24, 48 hours). Testis tissues fixed immediately were used as 0h controls. Viewing at 200x magnification allowed qualitatively assessment of the tissue for degradation and necrosis. at time points 6, 12, 24 and 48 hours culturing.

2.11 Explant Culture System: Exposure to Environmental Pollutants

Tissue was prepared for culturing as per optimization and tissue samples were cultured for 24 hours in the presence of Luteinizing hormone (LH)(pLH – Sigma Aldrich) or Follicle Stimulating hormone (FSH)(pFSH – National Hormone and Peptide Programme, Torrance, CA) at 0.0, 1.0, 10.0, 100.0ng/ml. Each plate had one positive control well in which tissue was cultured with Dibutyryl cyclic- Adenosine monophosphate (Db cAMP) (Sigma Aldrich – Product Number D0627) (10 mM/ml). This substance, an analogue for the cAMP signaling molecule, confirms testosterone production capabilities of the tissue in the culture system by effectively bypassing LH-ligand interaction (Laskey & Phelps, 1991).

For culturing with chemical pollutants, PCB 153 and DEHP were dissolved in a 0.01% DMSO solution as vehicle in the culture medium. Concentrations of PCB 153 (Sigma Aldrich) and DEHP (Sigma Aldrich) at 10x and 100x mean testes background concentrations determined from previous analysis of testes tissue (PCB 153 = 0.90 µg/kg; DEHP = 0.31 90 µg/kg) were used to culture the tissue. See figure 2.2.for plate layout.

After a 24 hour incubation period the supernatants were collected. These were frozen at minus 20°C in preparation for analysis for testosterone concentrations.

LH	1	2	3	4	5	6
A	-ve Control Medium	LH 0.0 ng/ml + VEHICLE	PCB Conc 1 LH 0.0 ng/ml	PCB Conc 2 LH 0.0 ng/ml	DEHP Conc 1 LH 0.0 ng/ml	DEHP Conc 2 LH 0.0 ng/ml
B	Db cAmp 10 mM/ml	LH 1.0 ng/ml + VEHICLE	PCB Conc 1 LH 1.0 ng/ml	PCB Conc 2 LH 1.0 ng/ml	DEHP Conc 1 LH 1.0 ng/ml	DEHP Conc 2 LH 1.0 ng/ml
C		LH 10.0 ng/ml + VEHICLE	PCB Conc 1 LH 10.0 ng/ml	PCB Conc 2 LH 10.0 ng/ml	DEHP Conc 1 LH 10.0 ng/ml	DEHP Conc 2 LH 10.0 ng/ml
D		LH 100.0 ng/ml + VEHICLE	PCB Conc 1 LH 100.0 ng/ml	PCB Conc 2 LH 100.0 ng/ml	DEHP Conc 1 LH 100.0 ng/ml	DEHP Conc 2 LH 100.0 ng/ml
FSH	1	2	3	4	5	6
A	-ve Control Medium	FSH 0.0 ng/ml + VEHICLE	PCB Conc 1 FSH 0.0 ng/ml	PCB Conc 2 FSH 0.0 ng/ml	DEHP Conc 1 FSH 0.0 ng/ml	DEHP Conc 2 FSH 0.0 ng/ml
B	Db cAmp 10 mM/ml	FSH 1.0 ng/ml + VEHICLE	PCB Conc 1 FSH 1.0 ng/ml	PCB Conc 2 FSH 1.0 ng/ml	DEHP Conc 1 FSH 1.0 ng/ml	DEHP Conc 2 FSH 1.0 ng/ml
C		FSH 10.0 ng/ml + VEHICLE	PCB Conc 1 FSH 10.0 ng/ml	PCB Conc 2 FSH 10.0 ng/ml	DEHP Conc 1 FSH 10.0 ng/ml	DEHP Conc 2 FSH 10.0 ng/ml
D		FSH 100.0 ng/ml + VEHICLE	PCB Conc 1 FSH 100.0 ng/ml	PCB Conc 2 FSH 100.0 ng/ml	DEHP Conc 1 FSH 100.0 ng/ml	DEHP Conc 2 FSH 100.0 ng/ml

FIGURE 2-1 PLATE LAYOUT FOR EXPLANT TISSUE CULTURE SYSTEM.

2.12 EIA TESTOSTERONE ASSAY

Supernatant from culture wells was collected and frozen at the end of the 24 hour incubation period then thawed at ambient temperature for the EAI assay. Supernatants were diluted in culture medium 200x in order to reduce testosterone concentrations so that they fell within the range of the EIA assay. Testosterone concentrations were measured in the thawed supernatants using a competitive enzyme-linked immunosorbent assay (EIA) method. Supernatant was incubated on a pre coated goat/anti-mouse IgG microtiter plate (Enzo Life Science, Farmingdale, NY) and then the yellow color generated measured on a microplate reader at excitation filter 405 nm, emission filter 620 nm (Fluostar Optima microplate reader, BMG LABTECH, Aylesbury, UK).

TABLE 2-6 EIA ASSAY FLOW CHART IDENTIFYING THE CONTENTS OF THE MICROTITER WELLS. TA: TOTAL ABSORPTION WELL, NSB: NONSPECIFIC BINDING , BO: MAXIMUM BINDING

Well ID	Blank	TA	NSB	B_O	Standard	Samples
Standard Diluent	200 µl	-	100 µl	100 µl	-	-
Assay Buffer	-	-	50 µl	-	-	-
Sample/Standard	-	-	-	-	100 µl	100 µl
Antibody	-	-	-	50 µl	50 µl	50 µl
Conjugate	-	5 µl	50 µl	50 µl	50 µl	50 µl
Substrate	200 µl	200 µl	200 µl	200 µl	200 µl	200 µl

Quality control measures necessitate the inclusion in the microtiter plate duplicate wells for all samples and the inclusion of a blank, total absorption (TA), nonspecific binding (NSB) and maximum binding (B_O) wells. Coefficient of variance(%) was calculated for all wells by the formula: St dev $_{Ave}$. $\%B_O$ / $_{Ave}$ $\%B_o$.

Total testosterone concentrations were done by calculating using the following formulae.

$_{Ave}$ OB – $_{Ave}$ NSB = $_{Ave}$ $\%B_O$

Standard curves were used to interpolate Log testosterone. These numbers were anti logged as total testosterone. Total testosterone was multiplied by the dilution factor (200X).

An adjusted per mg testosterone production was calculated by dividing tissue weight by the testosterone in each sample well.

Standard curves were created using culture medium with five testosterone concentrations (2000, 500, 125, 31.25, 7.81 pg/ml). Graphpad Prism was used to create standard curve graphs and interpolate testosterone values from the microtiter place optical density readings for sample wells (see Figure 2.2).

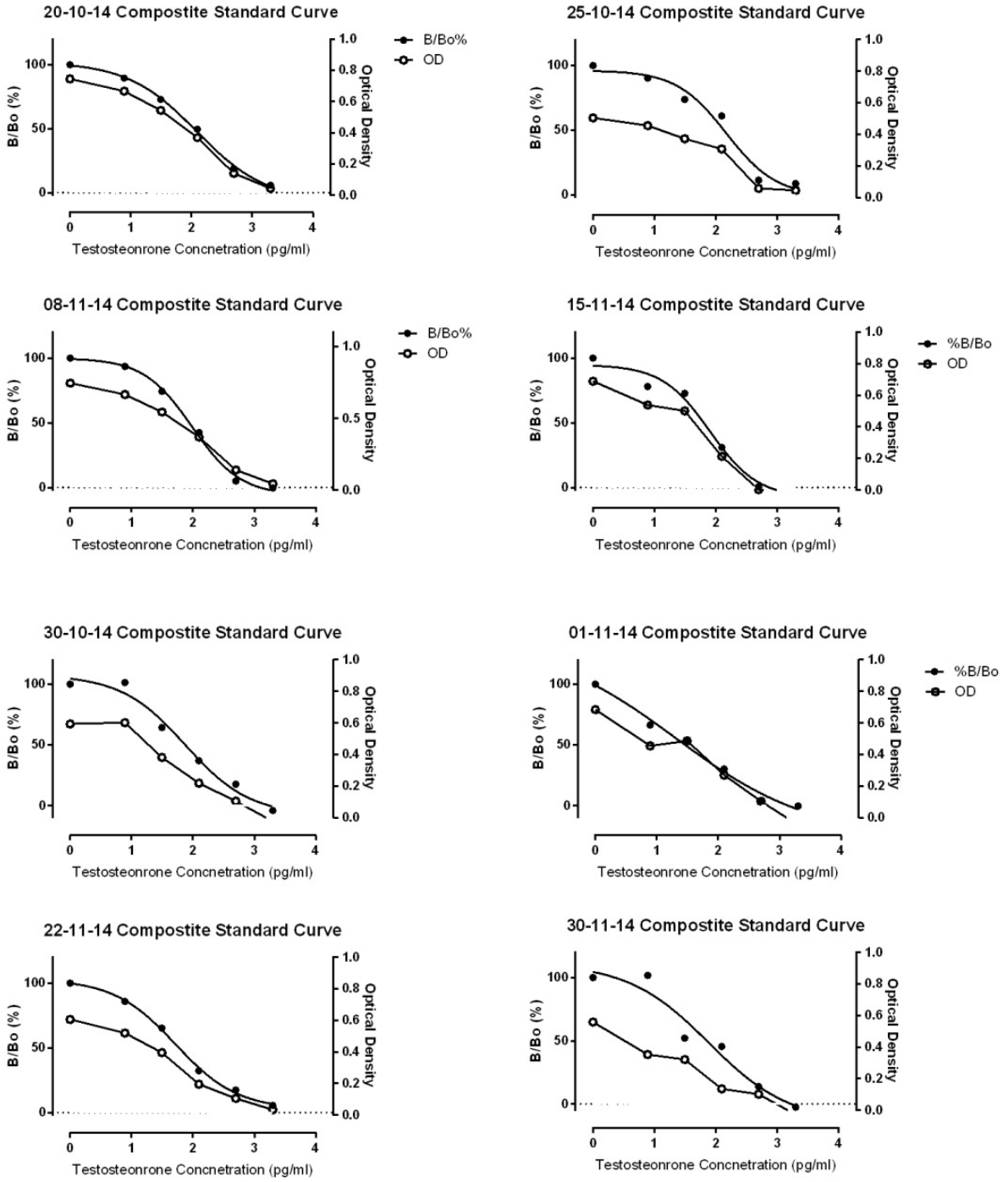


FIGURE 2-2 STANDARD CURVES USED FOR INTERPOLATING TESTOSTERONE CONCENTRATIONS FOR ALL EIGHT SAMPLES SHOWN BY DATE OF TISSUE COLLECTION.

2.13 DNA FRAGMENTATION/SPERM CHROMATIN DISPERSION TEST

Ejaculates were collected from stud dogs (n = 10) located at the Guide Dogs for the Blind National Breeding Centre (Leamington Spa). The collections were done and processed as described for previous sperm cultures (Section 2.2). Samples were shipped in extender (INRA 96, Nouzilly, France) via overnight post to the laboratory at University of Nottingham, School of Veterinary Medicine and Science, Sutton Bonington.

On arrival at laboratory, samples were assessed for motility (Total motility, Rapid Progressive motility, Non motile) based on a count of 100 individual sperm. The number of live and dead sperm was assessed using a Nigrosin-Eosin (Hancock) Stain as described previously based on a count of 200 individual sperm.

Mean background concentrations of PCB 153 and DEHP determined by previous analysis of adult canine testis (Mean PCB 153 = 0.90 µg/kg; DEHP = 0.31 90 µg/kg: Chapter 3.3.3) was used at 2, 10 and 100x concentrations to culture sperm. A simple Sperm Chromatin Dispersion (SCD) test was used to determine DNA fragmentation using bright field microscopy at 0 and 120 minutes. This was a modified methodology based on a commercially available kit (Vitrolife, Halosperm) and the methods of Fernandez (2005; 2003).

Semen samples were stored at 37°C and diluted down to ≈ 10 X 10^6 /ml. The lysis solution containing 1% Triton™ X-100 surfactant (Sigma Aldrich), 40 mMol Dithiothreitol (DDT), Sodium Chloride (2.5 mol) and Tris buffer (10 mMol) was warmed to room temperature. A 1% agarose solution was warmed to melt the gel and placed in a water bath at 37°C. A denaturing solution was prepared containing a 0.8mol hydrochloric acid solution.

An aliquot of the semen sample (20 µl) was mixed in an eppendorf tube with a measure of the agarose gel solution. A measure of the cell suspended agarose gel was placed on a microscope

slide and covered with a glass coverslip. The prepared slides were placed in a standard refrigerator at ≈ 5°C for five minutes minimum to set the agarose gel.

After removing slides from the refrigerator, coverslips were gently lifted off and the slides immersed in a denaturing solution (0.8mol hydrochloric acid) at room temperature for 7 minutes. After this incubation period, slides were gently washed for 5 minutes in distilled water.

Slides were progressively placed for 2 minutes in 70%, 90% and 100% ethanol solutions and allowed to dry. A Wright's Diff Quick stain was used to prepare the slide for bright field microscopy. The sperm were classified as either 'fragmented' or 'not fragmented' by examining the peripheral halo. Figure 2.3 shows stained spermatozoa positive for fragmentation (no halo) and alternatively sperm showing no fragmentation (halo). For the purposes of the assay, 200 individual cells were counted and identified as DNA fragmented or having no DNA fragmentation.

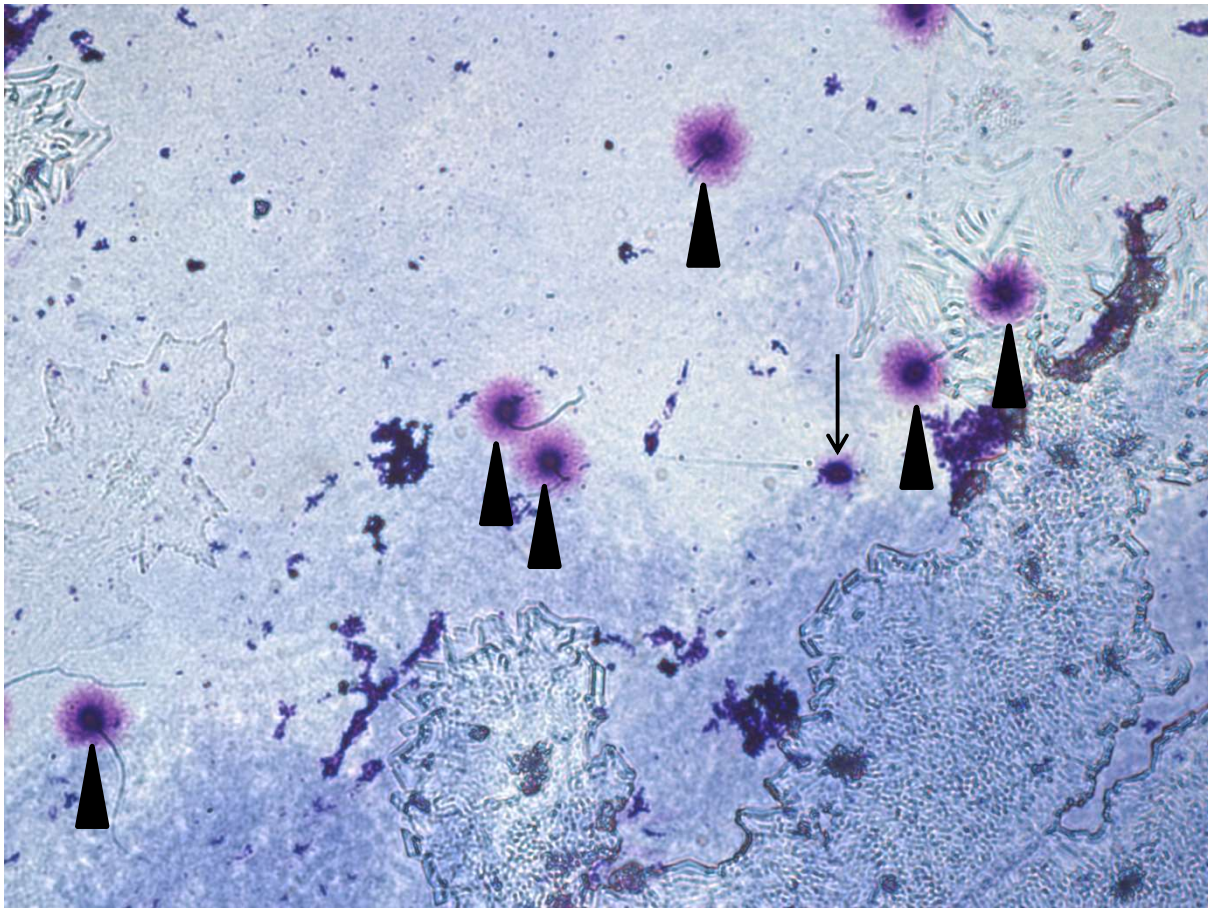


FIGURE 2-3 SPERMATOZOA STAINED WITH WRIGHT'S DIFF QUICK STAIN AND EXAMINED UNDER A BRIGHTFIELD MICROSCOPE (400X MAGNIFICATION). THE HALO GENERATED BY THE SPERM WITH NO DNA FRAGMENTATION IS INDICATED BY THE BLACK TRIANGLE SHAPE; THE DNA FRAGMENTED SPERM IS IDENTIFIED BY THE BLACK ARROW.

2.14 Statistical Analysis

A statistical model create by Professor Martin Green (University of Nottingham) was constructed to evaluate the change in semen quality over time whilst accounting for repeated semen measurements within dogs. Conventional multilevel models were specified (Dohoo, et al., 2003). Using MLwiN version 2.2222 with semen quality characteristics either modelled directly as a normal response variable or, when appropriate, log transformed, to ensure model residuals followed an approximately normal distribution. The effects on semen quality of age, breed (categorical variable), bodyweight and sire of dog (included as a random effect) were investigated. Since its inclusion had no substantive effect in any model, sire was not included as a random effect in the final models. Model specification was:

Semen Characteristic ij = $\alpha + \beta 1\text{-}n$ Year1-n + $\beta 2$ Xij + $\beta 3$ Xj + uj + eij

where the subscripts i, and j denote the ith measurement of semen quality (Percentage Normal Motility, Percentage Normal Sperm or Total Sperm Output), in the jth dog, α is the regression intercept, Year1-n are dummy variables representing each year of the 'n' study years, with $\beta 1\text{-}n$ the related coefficients, Xij are covariates associated with each semen quality reading (such as age of dog at sampling) with $\beta 2$ the related coefficients, Xj are covariates associated with each dog (such as breed) with $\beta 3$ the related coefficients, uj is a random effect to reflect residual variation between dogs (assumed to follow a Normal distribution with a mean of zero and variance Ωu) and eij is an error term reflecting residual variation between semen quality measurements (assumed to follow a Normal distribution with a mean of zero and variance Ωe).

Model building followed an exploratory stepwise procedure and explanatory covariates remained in the model when the significance probability was <0.05. Interaction terms between covariates were tested in the model and also remained when P<0.05. Model fit was visually assessed using graphs of residuals at each hierarchical level. The data were examined for points or higher level units with a large influence on parameter values and the models were re-run when necessary, with these points omitted, to examine their effect on parameter values.

Statistical analysis was performed using GenStat Discovery Edition 4 statistical analysis package (VSN International) and/or GraphPad Prism 6.07 Windows (GraphPad Software, La Jolla California USA, www.graphpad.com). The associations were analysed using non parametric Spearman's rank correlation coefficient with GenStat software. The P value is calculated as exact probabilities and the t value for correlations is adjusted for ties. The assumptions for ANOVA of independent Normally-distributed errors were confirmed by examination of Genstat's residual plots. Chemicals (PCB153, DEHP) were tested singly and in combination as a 2 chemical by 3 concentration by 4 times split plot factorial design. Raw data collected from the Hobsons Sperm Tracker was used and comparisons made using a general ANOVA model. Data was fitted to the model so that interactions between the concentration of each chemical and time could be assessed. Dogs were considered to be replicate blocks and sperm samples within dogs as the main plots. The main effect of concentration group was tested against the main-plot between-sample error; all other factors and their interactions were tested against the residual sub-plot error. ANOVA was conducted using GraphPad software as was unpaired T tests (two way) for examining differences. Means and standard

error of the difference values were plotted or expressed in the text and the threshold for statistical significance for P-values was 0.05.

Chapter 3 DECLINING MALE FERTILITY, REPRODUCTIVE HEALTH & CHEMICAL EXPOSURE

3.1 INTRODUCTION

Human TDS includes a reduction in accepted indicators of fertility such as semen quality (Skakkebaek et al., 2001) and developmental perturbation leading to hypospadias and cryptorchidism as well as testis cancer (Toppari et al., 2010). Companion animals may be sentinels of exposure to environmental contaminants as through cohabitation with humans they are exposed to the same ubiquitous chemical pollutants with the same possible consequences for reproductive health. As there is a suggested environmental aetiology for human TDS (Fisher, 2004), companion animal fertility, due to a shared environment, warrants investigation. There is no published evidence which identifies temporal trends in companion animal fertility and reproductive health. Indeed, if similar negative trends to those identified in humans could be shown in companion animals, the dog would prove to be a useful experimental/sentinel model. This is especially significant if the hypothesis of an association between environmental pollution and reproductive impairment is accepted.

3.1.1 A PROPOSED CANINE TDS

The work of Carlsen et al. (1992) and Swan (Swan et al., 2000), based on meta-analysis research, suggested a decline in human male fertility and sperm quality. This seminal work instigated subsequent research into temporal trends of male sperm quality and related aspects of reproductive health and semen quality. Thus semen quality became a primary factor in the testicular dysgenesis syndrome (Bay et al., 2006) along with developmental impairment (cryptorchidism, hypospadias) and testis cancer. As a key manifestation of human TDS, temporal trends in semen quality are one of the most difficult to validate as methodological differences in assessment techniques have occurred (Pacey, 2013). If negative temporal trends in semen quality are evidenced in the dog, this would contribute to the hypothesis that semen quality is declining is due to an environmental aetiology.

When looking for a model to provide evidence of negative temporal trends in semen quality beyond the human, there is a paucity of published work. Soon after the publication of Carlsen's 1992 work a research group examined historic data held on a range of production animals (Setchell, 1997) over a similar time period. This group suggested no overall negative trends in semen quality of three farm species (bull, boar and ram). This conclusion was seemingly contradicted by subsequent research. Wahl and Reif (2008) demonstrated a decline in semen quality in a group of bulls over a 30 year period from 1965. These few reports on temporal trends in semen quality in production animals must be considered along with the knowledge that trends in semen quality are affected by selective breeding practices. Also, human population dynamics have shown a general migration from rural to urban areas (Setchell, 1997) but farm animals obviously remain strictly in rural areas. Companion animals follow similar patterns to humans but the work with production animals presents the two mentioned considerations: urban vs rural residency and selective breeding practices.

Although, published evidence of impaired fertility or reproductive dysgenesis in companion animals, specifically canidae and felidae, is limited there is some evidence that over the last 40 years the incidence of testicular tumours seen in dogs has increased in parallel with temporal trends seen in humans (Grieco et al., 2008). Since environmental factors have been linked to an increased incidence of human testicular cancer perhaps similar environmental factors may account for the rise in incidence of testicular cancer in dogs. In support of this hypothesis, histological signs which characterise the early stages of human TDS, testicular seminomas (Looijenga, 2009), have recently been described in the dog (Grieco et al., 2008). These researchers identified seminiferous tubule abnormalities and germ cell neoplasia *in situ* (GNIS) cells in canine testes. Since GNIS cells are known precursors to human seminomas (Looijenga, 2009) TDS is also likely to predispose the canine testis to cancer and have a shared aetiology.

There is no published evidence which identifies temporal trends in companion animal fertility, particularly in relation to semen quality. This is in part because monitoring of semen quality is not routinely done with companion animals. One exception to this practice would be the practices found in breeding centres of service animals such as the Guide Dogs for the Blind Association. Fertility information may become more available for dogs as artificial reproductive technologies (ART), such as semen cryopreservation, are becoming more commonplace (Sirivaidyapong et al,, 2001). Along with these procedures, ART, comes recognition of the value of fertility assessment (Root Kustritz, 2007) and the standardisation of these procedures (Smith & England, 2001; Kutzler, 2005). These standard protocols are crucial for examining temporal trends because, as previously stated, the credibility of the human research into temporal trends has been held in question due to nonstandard laboratory practice over time.

In order to investigate the possibility of canine TDS, in addition to examining semen quality and other aspects of TDS in companion animals, the environmental aetiology as suggested for

humans needs to be investigated. Companion animals have been suggested by other researchers as a valid model for research into chemical pollutants (van der Schalie et al., 1999; Backeret al., 2001) which are linked to a decline in reproductive health.

3.1.2 Evidence of canine exposure to EDs and other environmental pollutants

If a canine TDS is hypothesised, this would have to extend beyond its immediate manifestations, related to fertility and reproductive health, towards evidence of an environmental aetiology. This evidence would be found in exposure to chemical pollutants which are known reproductive toxins, found in the dog's environment and diet. Although this would not definitively prove causation, a demonstrated association with chemical pollutants would give it equal status to what has been reported in the human. Furthermore, the general exposures through absorption and inhalation could be similar to those found in the shared environment between dogs and humans. The difference between human vs canine exposures that warrants detailed investigation would be diet, as this is a specific discrepancy between the exposures between species. The variation in dietary exposure for dogs is more likely to come from batch variation in a specific food brand than across a varied diet as general husbandry practices dictate consistency in feeding. As well as a determination of chemical pollutant concentrations in the dog's diet, evidence that these chemical pollutants have concentrated in the dog's tissue and body fluid media would need to be determined and associated directly with tested food brands.

Concentrations of EDs have been found in companion animals and this contamination may be via a dietary route. Bisphenol-A (BPA) has been detected in fifteen commercially available cat foods and 11 commercially available dog foods (Kang & Kondo, 2002). Further research, using a narrower range of commercially available pet foods also found BPA present in some samples but at lower concentrations (Schecter et al., 2010). Additional studies report the presence of

other contaminants in pet food including PCBs, DDE and heavy metals (Furr et al., 1976; Mumma et al., 1986; Sakai et al., 1995; López-Alonso et al., 2007). Moreover, possible implications of these detected concentrations of contaminants have raised concerns to the extent that owners are recommended to reduce their pet's consumption of canned food (Edinboro et al., 2004).

Diet has been established as a primary route for contamination of certain EDs (e.g. BPA) in humans and particularly in human children (Wilson et al., 2007; Pinto & Carvalho, 2013) as well as for companion animals as demonstrated by the work of Verreault et al. (2008). These researchers fed captive sled dogs a diet of porcine fat (control) or a diet of Minke whale blubber (treatment) and measured a range of EDs in the test animal's tissue (See Table 3.1.). A clear correlation was found between high concentrations of organohalogen contaminants in the Minke Whale blubber and in adipose tissue from dogs on this diet, as well as their pre and post-weening pups. Of particular note is that these findings equate with concentrations of environmental contaminants measured in Greenland people eating a traditional diet which includes marine mammals (Bonefeld-Jorgensen, 2010).

TABLE 3-1 ORGANOHALOGEN CONTAMINANT CONCENTRATIONS (MEAN ± STANDARD ERROR) IN ADIPOSE TISSUE OF TWO SAMPLE GROUPS OF CAPTIVE SLED DOGS FED TWO EXPERIMENTAL DIETS BASED ON EITHER PORCINE FAT (CONTROL) OR MINKE WHALE BLUBBER (TREATMENT)(VERREAULT ET AL., 2008).

Organohalogen	Control	Treatment
ΣPBDE(ng/g wet wt.)	2.1±0.4	41.9±0.7
HCB(ng/g wet wt.)	N.D.	18.6±0.3
ΣCHL(ng/g wet wt.)	N.D.	196±5.4
ΣPCB(ng/g wet wt.)	N.D.	1147±58.8

N.D: Not Detected
ΣPBDE: sum of 36 polybrominated diphenyl ether congeners
HCB: hexachlorobenzene
ΣCHL; sum of 6 chlordane compounds
ΣPCB: 59 polychlorinated biphenyl congeners

Concentrations of environmental pollutants known to disrupt reproductive development and function have been found in both cats and dogs. Table 3.2 illustrates that a range of endocrine disrupting chemicals have been detected in both dog and cat tissue and serum. The flame retardant PBDE has been detected in serum from cats and dogs and both studies suggest that these species may be good sentinels of human exposure (Dye et al., 2007; Venier & Hites, 2011). Interestingly in the feline study, serum concentrations of PBDE have been associated with feline hypothyroid disease and concentrations of PBDE in the sample animals were comparable to relatively high concentrations found in human infants (Frederiksen, Vorkamp, Thomsen, & Knudsen, 2009). The latter observation, attributed to proximity to household materials treated with PBDE based flame retardants and higher concentrations of dust consumption, suggests that these species may be sentinels of exposure for human infants.

TABLE 3-2 EXAMPLES OF EDS CONCENTRATIONS FOUND IN COMPANION ANIMALS.

Companion Species	ED CATEGORY	∑/congener	Concentrations (mean)	Reference
Canine	PAH	BaA	0.19ng/g	(Takagi et al., 2005)
		BkF	0.08ng/g	
		BaP	0.1 ng/g	
		BghiP	0.1 ng/g	
	Dioxin	PCDD	6.0pg/g	
		PCDF	1.12pg/g	
		DL-PCB	70.2pg/g	
Feline	PBDE	∑	9.78±2.07ng/g*	(Dye et al., 2007)
Feline	DDT	*p,p*-DDE	94.75ng/g	(Storelli et al., 2009)
	PCB	∑	199.02ng/g	
Canine	PCB	∑	41.61ng/g	
Canine	PBDE	∑	3.96±2.23ng/g*	(Verier & Hites, 2011)
Canine	PCB	∑	Range: 50 – 2995ng/g	(Kirkegaard et al., 2011)
Feline	PBDE	∑	4505 ± 1006ng/g*	(Guo et al., 2012)
	PCB	∑	203 ± 29.4ng/g*	

In addition, polychlorinated biphenols (PCBs) have been detected in adipose tissue from cats and dogs (Storelli et al., 2009). Interestingly, higher concentrations in cats were attributed to the better ability of dogs to metabolise some of these contaminants. This could also be attributed to higher concentrations of dust consumption by cats during grooming behaviour. However, if metabolic differences are responsible for the discrepancy in somatic burden of EDs between companion species, the cat may be a better sentinel for overall environmental contamination whilst the dog is a better indicator of bioaccumulation of EDs in humans.

In order to establish a companion animal model of environmental pollutant effects on reproductive development and function (leading to TDS as seen in humans) three important factors must be considered: (1) is there any evidence of negative temporal trends in factors of reproductive development and fertility in companion animals? (2) can this be demonstrated for more than one component of TDS as defined in the human? and (3), are environmental pollutants present in the animal's environment and soma? Although some evidence of an increase in testis cancer and GNIS cells has been there is little evidence of other

manifestations of TDS in companion animals. Environmental pollutants have been found in the tissue and serum of cats and dogs but this research is limited to testing single media. Semen quality in humans largely instigated the investigation into TDS and is one of the most important markers of reproductive function. A parallel investigation into a companion animal species has not been undertaken. Additionally there has not been research using companion animals to identify concentrations of environmental pollutants in the animal's environment (specifically diet), serum and tissue.

The aims of this chapter are to determine if the key manifestations of human TDS are also present in the dog. Specifically, parameters related directly to temporal trends in sperm quality and developmental disorders will be examined. Additionally, investigations will be carried out to determine if EDs are detectable in canine diet, tissue or other somatic media.

Towards these aims the following objectives have been identified:

1. Determine if there is a temporal trend in parameters of canine semen quality in a population of breeding dogs affiliated to the Guide Dogs for the Blind Association.
2. Evaluate records, maintained in a details database held on the same breeding population of dogs, and determine if the incidence of cryptorchidism in pups has changed over time.
3. Determine which EDs presence and concentration in a range of canine food, tissue and somatic media, including testes, prostatic fluid, milk, full ejaculate.

3.2 METHODS

All aspects of the study were approved by and meet the ethical requirements of the University of Nottingham, School of Veterinary Medicine and Science.

Data on canine fertility of a cohort of dogs as part of a national service dog breeding programme was provided for identification of temporal trends. Full details of the data collection are provided in Chapter 2.4. It is important to emphasise that all semen was collected and evaluated by a single experienced person (Professor Gary England) or one of 3 technicians trained by him.

The incidence rate of cryptorchidism in puppies from 1995 to 2014 was assessed from records maintained on the National Breeding Centre database (average number of male dogs per year = 651.25±14.79). The number of cases of cryptorchidism was calculated showing an incidence rate per 100 dogs. This is based on the number of animals identified in the data base as cryptorchid and the number of male pups born that year. Additionally, sex ratios were calculated on this same group of dogs born during the same time period (male:female ratio = number of males/total pup number per litter).

A range of commercially available dog foods (n = 28) was tested for three representative EDs: Polychlorinated biphenyls (PCB), Polybrominated Diphenyl Ethers (PBDE) and Di(2-ethylhexyl)phthalate (DEHP). Fourteen samples of dry dog food and 14 samples of canned food was tested for PCBs (congeners 28, 47, 101, 118, 138, 153, 180), PBDEs (congeners 28, 47, 99, 100, 153, 154, 183) and DEHP. Details of chemical testing methodologies are provided in Chapter 2.3.

Details of the method of collection of data and tissue are provided in Chapter 2.2 and the questionnaire used to record individual dog's information is found in Appendix B.

The tissue was prepared for chemical testing and histological assessment at the point of collection by the veterinary surgeons and other staff undertaking the castrations. These staff followed the directions provided with the tissue collection questionnaire.

Prostatic fluid was collected as residual material during routine fertility assessment of dogs involved in the GDBA breeding programme. Sperm rich fractions (fraction 2) of 14 canine ejaculates were collected and two pools generated of 5 samples and one of 4 samples for chemical testing. Canine bitches milk was collected from lactating bitches involved in the GDBA breeding programme. All samples were frozen (-20°C) prior to being shipped for chemical testing. Chemical analysis was conducted at the James Hutton Institute using methods described in Chapter 2.3.

A statistical model was constructed by Professor Martin Green (University of Nottingham) to evaluate the change in semen quality over time whilst accounting for repeated semen measurements within dogs. The analysis utilised conventional multilevel models and full details are provided in the statistical methods Section (2.14).

3.3 RESULTS

3.3.1 TEMPORAL TRENDS IN CANINE FERTILITY AND REPRODUCTIVE HEALTH

A decrease in the percentage of normal forward progressive motility sperm was observed between years 1988-1998 (decline = 1.3% per year) with years 1992-1996 and 1998 being significantly lower than 1988 (R^2=0.232:) (Figure 3.1A). After removal of dogs with the poorest semen quality in 1999 and 2000, there was a further decrease in motility (decline = 1.4% per year), with years 2007, 2010-14 being significantly lower than 2001 (Figure 3.1A). Between 1988 and 1998, the mean percentage of live normal sperm declined with years (1.3% per year) (F=0.00, R^2 =0.083) (Figure 3.1B). After removal of dogs with the poorest semen quality in 1999, there was a numeric decline in the Percentage Normal Sperm with time (Figure 3.1B), although only years 2004, 2007 and 2008 were significantly lower than 2001. An increase in the total sperm output was observed between years 1988-1998 with years 1992-1998 being significantly greater than 1988 (Figure 3.1C). After removal of dogs with the poorest semen quality in 1999, total sperm output (TSO) increased substantially, but over the following 10 year period there was a decline in TSO with time (Figure 3.1C) with 2002, 2003 and 2006-2010 being significantly lower than 2001. For all three parameters (percentage normal motility, percentage normal sperm and total sperm output), there were significant differences between breed and a non linear decline with age.

Analysis of records of cryptorchidism from the GDBA database revealed a significant change in the annual incidence of cryptorchidism over a seventeen year period from 1995 (Figure 3.1D). The annual incidence rate per year (average number of dogs born per year was 654 (SEM ± 14.71)) showed a significant regression equation indicating a

positive trend in the incidence rate of this developmental disorder (R^2 = 0.33; n = 20; P < 0.05).

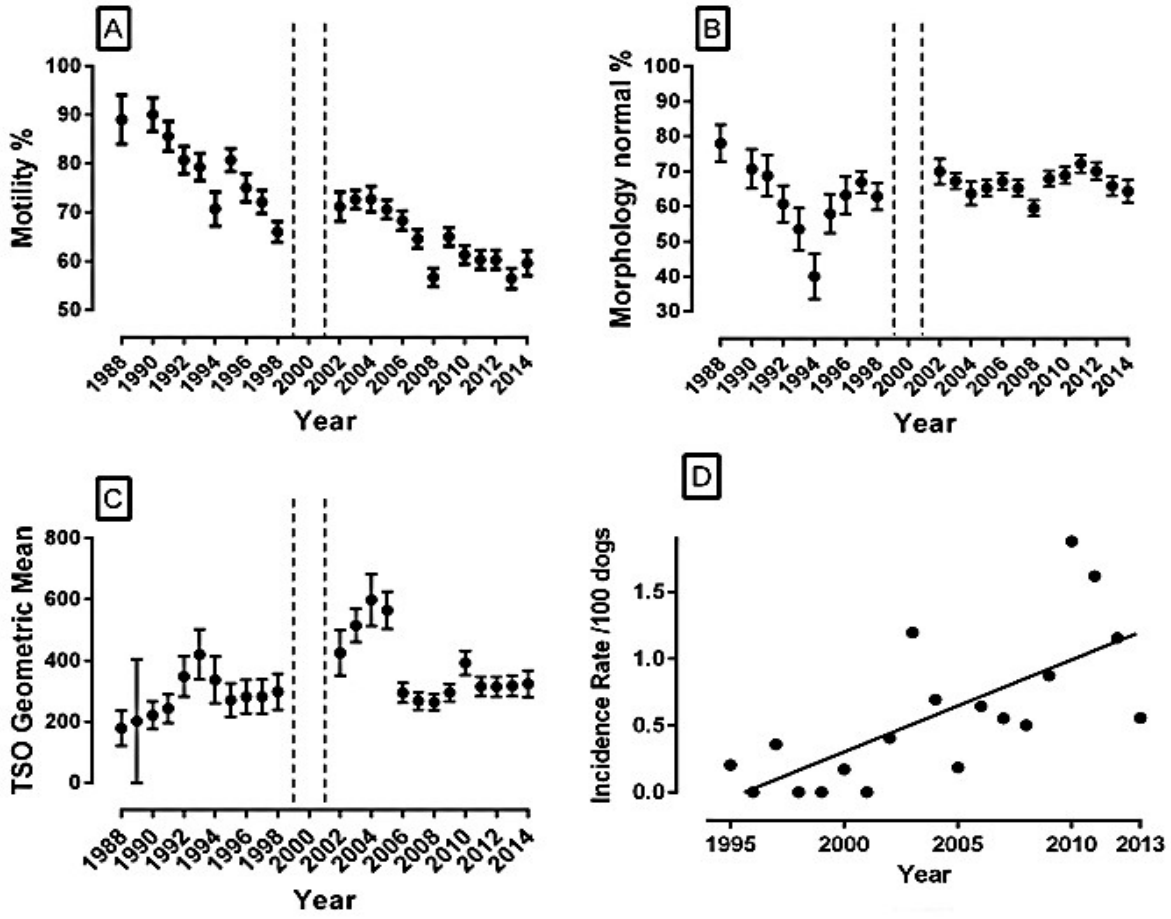


FIGURE 3-1 TEMPORAL CHANGES IN SEMEN QUALITY AND CRYPTORCHIDISM IN A POPULATION OF STUD DOGS. (A-C) GRAPHIC SUMMARIES OF THE FINAL MODEL PARAMETERS FOR (A) PERCENTAGE NORMAL MOTILITY, (B) PERCENTAGE LIVE NORMAL SPERM AND (C) TOTAL SPERM OUTPUT. IN 1999 TO 2000, DOGS WITH A DRAMATIC REDUCTION IN PERCENTAGE NORMAL MOTILITY WERE REMOVED FROM THE PROGRAMME. VALUES OF EACH PARAMETER ARE GIVEN FOR YEARS 1988-1998 AND YEARS 2001-2014 (N= 232 DOGS: 18-32 IN ANY ONE YEAR). (D) INCIDENCE RATE OF CRYPTORCHIDISM IN PUPPIES GENERATED AT THE NATIONAL BREEDING CENTRE FROM 1995 TO 2014 (N = 20; P = 0.057) ERROR BARS ON A-C REPRESENT ± THE STANDARD ERROR OF EACH ESTIMATED MEAN POINT VALUE FROM THE MODEL.

Two subsequent analyses demonstrated factors which are peripherally related to reproductive function. The sex ratio of male to female pups increased significantly (R^2=0.2; P<0.04) over the time period of 1995 – 2014 (Figure 3.2A). Over the same time period the survival of male pups increased significantly (P < 0.05) (Figure 3-2B).

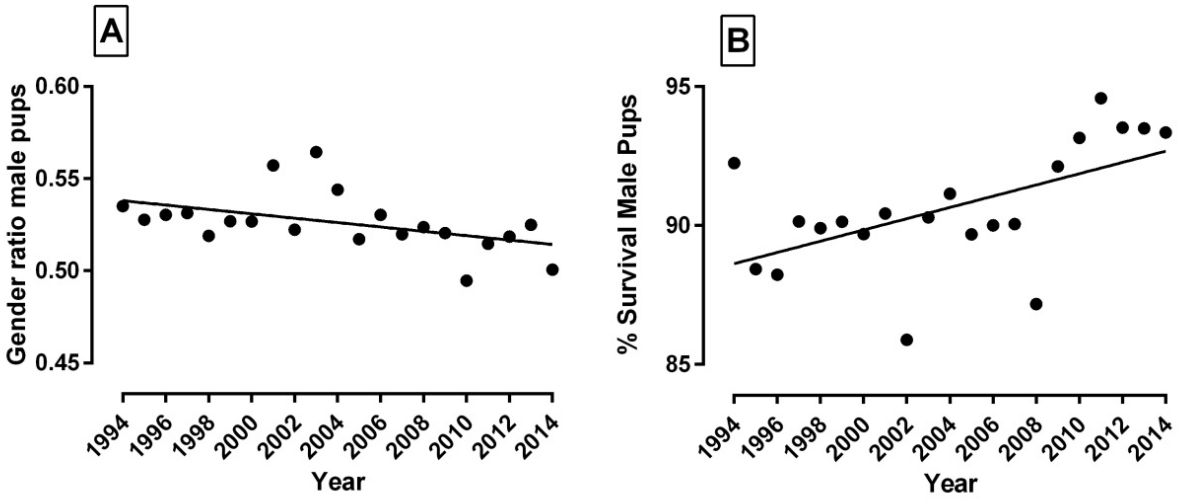


FIGURE 3-2 SEX RATIO AND SURVIVAL OF MALE PUPS BORN FROM 1994 – 2014 TO THE GDBA. A) SEX RATIO OF MALE PUPS (AVERAGE ANNUAL NUMBER OF MALE PUPS PER YEAR = 651 (1 SEM=14.79)) OVER THE TIME PERIOD 1994-2014. LINEAR REGRESSION LINE INDICATES A SIGNIFICANT DECREASE IN THE SEX RATIO OF MALE PUPS DURING THIS TIME PERIOD (N=21; R2=0.2; P=0.04). B) SURVIVAL OF MALE PUPS OVER THE SAME TIME PERIOD SHOWING A SIGNIFICANT INCREASE (N = 21; R2=0.31; P=0.0079) IN PERCENT OF MALE PUPS BORN ALIVE.

3.3.2 CONCENTRATIONS OF EDS IN CANINE DIET

All PCB congeners were detected in dog food samples. The frequency of occurrence (FO), samples reporting concentrations above the concentrations of detection, in dry food was 100% (n=14) for five congeners, with 92.9% and 85.7% for the remaining two congeners (PCB 52, 138). The wet food samples (n=14) also showed four of seven congeners with 100% frequency of occurrence with both remaining congeners at 92.9% (PCB 28, 101, 118). Frequency of occurrence for each PCB congener is show in in Table 3.3. The mean of ΣPCBs for wet and dry food was 1.35 (SEM± 0.5792) and 0.78 (SEM± 0.223) µg/kg respectively. The congener with the highest level in both wet and dry food was PCB153 with a mean level of 0.39(SEM± 0.193) and 0.22(SEM± 0.11) µg/kg respectively. Full results for all chemical concentrations found in dog food are shown in Table 3.3.

TABLE 3-3 CONCENTRATIONS OF ENVIRONMENTAL CHEMICALS IN SELECTED COMMERCIALLY AVAILABLE WET AND DRY DOG FOOD. MEAN VALUES WITH STANDARD ERRORS OF THE MEAN ARE SHOWN FOR EACH CHEMICAL CONGENER AS WELL AS MEDIAN AND ARITHMETIC RANGES. FREQUENCY OF OCCURRENCE (FO) IS SHOWN IN PERCENT. CONCENTRATIONS FOR PCB, PBDE SHOWN AS µG/KG AND DEHP IS SHOWN AS µG/G. LIMITS OF DETECTION: PCB = 0.02µG/KG, PBDE 28,47, 99, 100 = 0.02 µG/KG; PBDE 153,154, 183 = 0.5 µG/KG; DEHP = 0.05µG/G.

Food Type	Chemical/ Congener	Mean	SEM	Median	Range	FO%
WET	PCB 28	0.1143	0.01304	0.105	0.19	92.9
WET	PCB 52	0.1721	0.07793	0.05	1.03	100
WET	PCB 101	0.1464	0.05877	0.105	0.85	92.9
WET	PCB 118	0.1457	0.0733	0.065	1.06	92.9
WET	PCB 138	0.2693	0.1388	0.12	2.01	100
WET	PCB 153	0.3943	0.193	0.185	2.81	100
WET	PCB 180	0.1064	0.04058	0.055	0.58	100
WET	ΣPCB	1.345	0.5792	0.635	8.35	100
DRY	PCB 28	0.08571	0.01166	0.075	0.15	100
DRY	PCB 52	0.09167	0.03293	0.035	0.33	85.1
DRY	PCB 101	0.08214	0.03245	0.03	0.43	100
DRY	PCB 118	0.1057	0.03808	0.06	0.54	100
DRY	PCB 138	0.1377	0.05316	0.06	0.69	92.9
DRY	PCB 153	0.2179	0.07631	0.11	1.03	100
DRY	PCB 180	0.07643	0.02165	0.045	0.3	100
DRY	ΣPCB	0.7764	0.223	0.515	3.14	_
WET	PBDE 28	0.003571	0.002482	0	0.01	14.3
WET	PBDE 47	0.3386	0.03947	0.325	0.55	100
WET	PBDE 99	0.05786	0.01645	0.05	0.18	64.3
WET	PBDE 100	0.01429	0.008753	0	0.08	21.4
WET	PBDE 153	0.005	0.003437	0	0.01	14.3
WET	PBDE 154	0.008571	0.004671	0	0.02	21.4
WET	PBDE 183	0.003571	0.003571	0	0	7.1
WET	ΣPBDE	0.025	0.005	0.025	0.01	_
DRY	PBDE 28	0.004286	0.001727	0	0.01	35.8
DRY	PBDE 47	0.3021	0.03117	0.31	0.45	100
DRY	PBDE 99	0.002857	0.002857	0	0	7.1
DRY	PBDE 100	0.5129	0.1007	0.44	1.26	92.9
DRY	PBDE 153	0.01357	0.0052	0	0.02	35.7
DRY	PBDE 154	0.01	0.006112	0	0.05	21.4
DRY	PBDE 183	0.05857	0.02369	0	0.17	35.8
DRY	ΣPBDE	0.9057	0.1231	0.83	1.71	_
WET	DEHP	0.3731	0.1031	0.21	1.29	92.9
DRY	DEHP	0.2043	0.02711	0.165	0.33	100

All seven PBDE congeners were found in concentrations above the limits of detection across the food samples. Although only PBDE 47 was present at detectable concentrations in all food samples tested (FO = 100%). Congener PBDE 47 showed the highest relative mean concentrations in wet food (0.3386µg/kg (SED± 0.03947)) and congener PBDE 100 in dry food (0.5129µg/kg (SED± 0.01007). Table 3.3 shows the FO and mean values found in dog food for all congeners.

DEHP results showed a FO for wet and dry food as 92.9 and 100 per cent respectively. DEHP was found in all samples of wet food and the mean concentrations in these samples was 0.37µg/g as compared to 0.20µg/g in the dry food but this difference was non-significant ($P > 0.05$).

A comparison of the different concentrations of these chemicals between adult and puppy food is demonstrated in Figure 3.3 A - E with the differences between the congeners of interest (PCB 153 and PBDE 47) showing no significant difference between the food types (Figures 3.3D-E). DEHP also showed no significant difference in the mean concentrations found in puppy food and adult food (Figure 3.3C).

The difference in the concentrations of ΣPCB and ΣPBDE in wet and dry food was not significant for both chemicals ($P > 0.05$). Individual congeners of interest (PCB 153 and PBDE 47: those indicated in blue in Figure 3.4) reported relative high mean values in both food and testes. These two congeners (PCB 153, PBDE 47) also reported a frequency of occurrence of 100% indicating that all samples tested showed concentrations above the limits of detection. There was no significant difference between concentrations of PCBs, PBDE or DEHP detected in wet and dry food ($P> 0.05$). The mean concentrations are displayed graphically in Figure 3.4. for all chemicals and congeners detected in food and testes tissue.

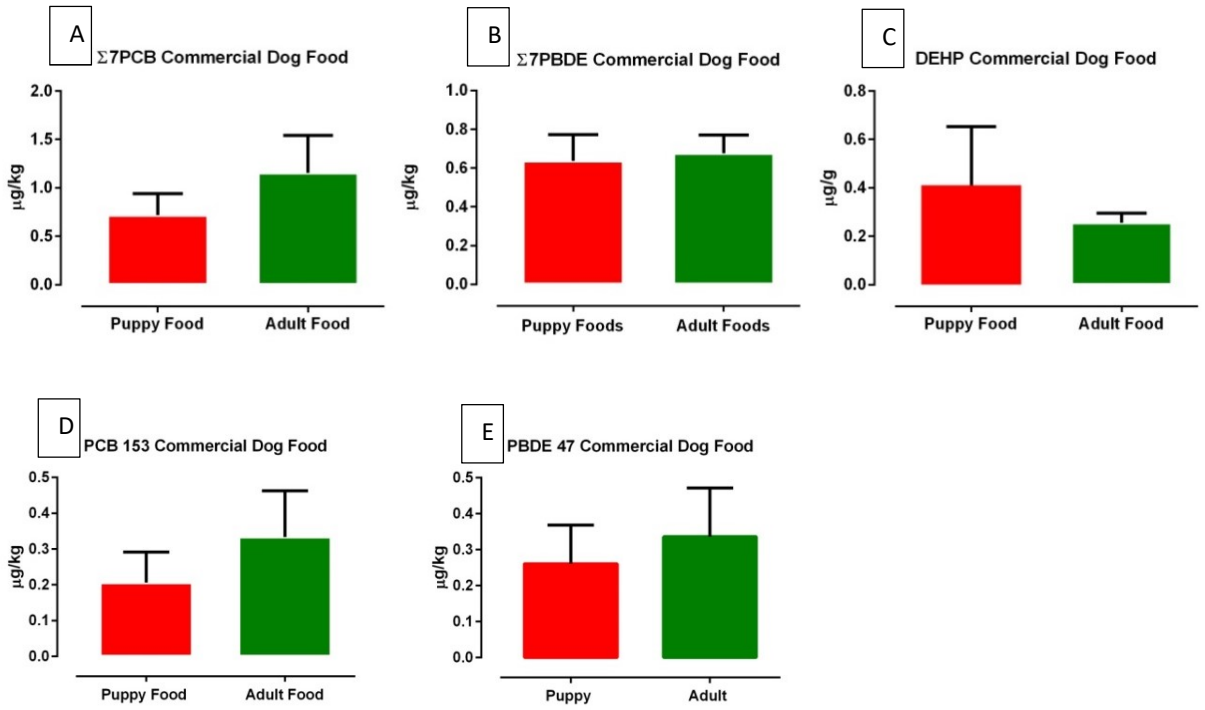


FIGURE 3-3 CONCENTRATIONS OF PCB, PBDE AND DEHP IN COMMERCIALLY AVAILABLE PUPPY FOOD VS ADULT FOOD IN THE 14 SAMPLES TESTED. (A – B) THE SUM OF 7 CONGENERS OF BOTH PBDE AND PCB SHOWED NO SIGNIFICANT DIFFERENCES BETWEEN THE CONCENTRATIONS WAS FOUND, (C) DEHP CONCENTRATIONS WERE NOT SIGNIFICANLY DIFFERENT BETWEEN PUPPY FOOD AND ADULT FOOD. (D – E) THE INDIVIDUAL CONGENERS OF PCH 153 AND PBDE 47 SHOWED NO SIGNIFICANT DIFFERENCE IN CONCENTRATIONS (P > 0.05).

3.3.3 CONCENTRATIONS OF EDS IN CANINE TESTES SAMPLES

The seven PCB congeners selected for testing all showed concentrations in canine testes tissue with three congeners reporting a FO of 100% (PCB 28,153, 180). The highest mean value was congener PCB 153 with concentrations of 0.3252µg/kg (SED± 0.05057). The congener (PCB 52) with the lowest mean value had a concentration of 0.06584 µg/kg (SED± 0.01744) and a FO of 62.857%.

PBDE 47 had the highest mean value of the seven congeners with a mean value of 1.878 µg/kg (SED ± 0.00539). This was also one of only two congeners having a FO of 100% (the other being PBDE 183). Two congeners (PBDE 153, 154) had concentrations below the limits of detection for all samples tested (FO = 0%).

DEHP was detected in all samples with a subsequent FO of 100%. The mean concentration of DEHP was 0.9377µg/g (SED ± 0.2261). Values for all congeners and chemicals reported in testes tissue are shown in Table 3.4.

TABLE 3-4 CONCENTRATIONS OF ENVIRONMENTAL CHEMICALS IN A COHORT OF UK CANINE TESTES. MEAN VALUES WITH STANDARD ERRORS OF THE MEAN ARE SHOWN FOR EACH CHEMICAL CONGENER AS WELL AS MEDIAN AND ARITHMETIC RANGES. FREQUENCY OF OCCURRENCE (FO) IS SHOWN IN PERCENT. CONCENTRATIONS FOR PCB, PBDE SHOWN AS µG/KG AND DEHP IS SHOWN AS µG/G.DRY MATERIAL. LIMIT OF DETECTION: PCB = 0.02µG/KG, PBDE 28,47, 99, 100 = 0.02 µG/KG; PBDE 153,154, 183 = 0.5 µG/KG; DEHP = 0.05µG/G.

Chemical Congener	Mean	SEM	Median	Range	FO%
PCB 28	0.4488	0.09266	0.2619	2.13845	100
PCB 52	0.06584	0.01744	0.0326	0.4433	62.857
PCB 101	0.3197	0.05846	0.2504	1.637	94.285
PCB 118	0.1593	0.02133	0.1325	0.5012	94.285
PCB 138	0.1509	0.03001	0.08207	0.8956	91.428
PCB 153	0.3251	0.05057	0.2308	1.32762	100
PCB 180	0.1999	0.03558	0.132	1.029292	100
ΣPCB	1.669	0.2695	1.112	6.7713	100
PBDE 28	0.04013	0.00539	0.03842	0.1166	100
PBDE 47	1.878	0.2198	1.704	5.7691	71.4
PBDE 99	0.1147	0.03483	0.05699	1.049	45.7
PBDE 100	0.118	0.03725	0	0.8085	0
PBDE 153	0	0	0	0	0
yPBDE 154	0	0	0	0	2.9
PBDE 183	0.04945	0.04945	0	1.731	100
ΣPBDE	2.201	0.2402	1.922	6.1538	100
DEHP	0.9377	0.2261	0.385	4.27	100

Certain congeners showed relatively high values in both food and testes tissue. These are identified graphically in Figure 3.4 with blue highlighting. PCB congener 153 had the highest concentration in testes, dry dog food and wet dog food. The PBDE 47 congener had the highest concentration in testis and canned dog food. In dry dog food, this congener (PBDE 47) was the second higher (followed by PBDE 100: mean 0.5129 (SED ±0.1007)). Figures 3.4 C, F, I highlight concentrations of DEHP in blue to indicate this chemical was found in concentrations across both food types and testes tissue.

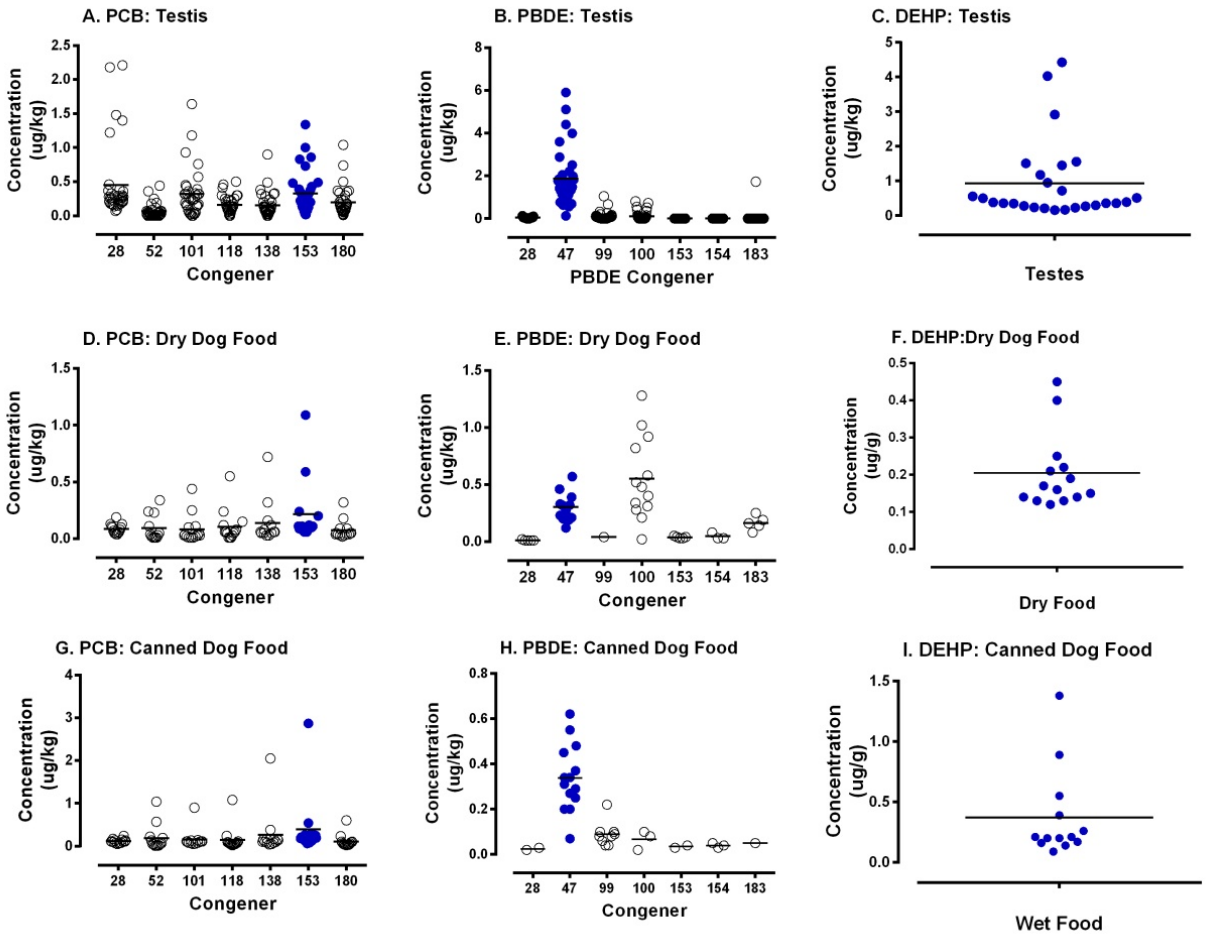


FIGURE 3-4 CONCENTRATIONS OF ENVIRONMENTAL CHEMICALS IN ADULT DOG TESTES AND COMMERCIALLY AVAILABLE DOG FOOD . (A,D.G) PCB CONGENERS, (B,E,H) PBDE CONGENERS AND (C,F,I) DEHP WERE MEASURED IN (A-C) TESTES, (D-F) DRY DOG FOOD AND (G-I) CANNED DOG FOOD. EACH CIRCLE REPRESENTS A SINGLE TESTIS OR FOOD SAMPLE. BLUE CIRCLES SHOW CHEMICALS THAT WERE DETECTED WITH GREATER CONSISTENCY ABOVE BASE CONCENTRATIONS IN TESTES AND FOOD. CONCENTRATIONS FOR PCB, PBDE SHOWN AS µG/KG AND DEHP IS SHOWN AS µG/G.DRY MATERIAL.

3.3.4 CONCENTRATIONS OF EDS IN PROSTATIC FLUID

The frequency of occurrence for the seven PCB congeners ranged from 7.14% (PCB 138, 153, 180) to 64.29% (PCB 28) with all seven congeners detected across the group. The highest mean (n = 13) concentration was found with congener PCB 28 (0.0286 µg/kg (SEM ± 0.0084)) and three sample at 0.0021 µg/kg were the lowest concentrations for the seven congeners. Five of the samples (PBDE 29, 100, 153, 183) showed no PBDE concentration above the limits of detection. Only two congeners (PBDE 47,99) showed concentrations above the limits of detection with a FO of 71.4% and 7.14% respectively.

The mean concentration detected for PBDE 47 was 0.0857 µg/kg (SEM ± 0.01901) and PBDE 99 recorded as 0.03571µg/kg (SEM ± 0.003571). It is worth noting that the mean for PBDE 47 represents only one sample showing a concentration above the limit of detection. Table 3.5 shows results for all chemicals in canine prostatic fluid.

TABLE 3-5 CONCENTRATIONS OF ENVIRONMENTAL CHEMICALS IN CANINE PROSTATIC FLUID (N = 13). MEAN VALUES WITH STANDARD ERRORS OF THE MEAN ARE SHOWN FOR EACH CHEMICAL CONGENER AS WELL AS MEDIAN AND ARITHMETIC RANGES. FREQUENCY OF OCCURRENCE (FO) IS SHOWN IN PERCENT. CONCENTRATIONS FOR PCB, PBDE SHOWN AS µG/KG AND DEHP IS SHOWN AS µG/G.DRY MATERIAL. LIMITS OF DETECTION: PCB = 0.02µG/KG, PBDE 28,47, 99, 100 = 0.02 µG/KG; PBDE 153,154, 183 = 0.5 µG/KG.

Chemical/ Congener	Mean	SEM	Median	Range	FO%
PCB 28	0.0286	0.0084	0.0200	0.1000	64.29
PCB 52	0.0043	0.0023	0.0000	0.0200	21.43
PCB 101	0.0050	0.0027	0.0000	0.0300	21.43
PCB 118	0.0036	0.0025	0.0000	0.0300	14.29
PCB 138	0.0021	0.0021	0.0000	0.0300	7.14
PCB 153	0.0021	0.0021	0.0000	0.0300	7.14
PCB 180	0.0021	0.0021	0.0000	0.0300	7.14
ΣPCB	0.0479	0.0184	0.0250	0.2400	-
PBDE 28	0.0	0.0	0.0	0.0	0.0
PBDE 47	0.0857	0.01901	0.11	0.19	71.4
PBDE 99	0.03571	0.003571	0.0	0.05	7.14
PBDE 100	0.0	0.0	0.0	0.0	0.0
PBDE 153	0.0	0.0	0.0	0.0	0.0
PBDE 154	0.0	0.0	0.0	0.0	0.0
PBDE 183	0.0	0.0	0.0	0.0	0.0
ΣPBDE	0.089281	0.01993	0.09	0.25	-

3.3.5 CONCENTRATIONS OF EDS IN CANINE MILK

All seven PCB congeners were detected in canine milk samples (n = 9). The highest concentration was PCB 28 with a mean level of 0.3792 µg/kg (SEM ± 0.109). PCB 153 was found in all samples (FO = 100%) at a mean level of 0.142 µg/kg (SEM ± 0.0586). The congener found at the lowest concentration was PCB 52 (mean 0.006248 µg/kg) which was only found in one sample (FO = 11.1%) with an individual concentration of 0.06 µg/kg. This was also one of the pooled samples. Table 3.6 displays all results for concentrations of PCB congeners found in canine milk.

TABLE 3-6 CONCENTRATIONS OF ENVIRONMENTAL CHEMICALS IN CANINE MILK. MEAN VALUES (N = 9: 2 POOLED SAMPLES OF 2 DOGS) WITH STANDARD ERRORS OF THE MEAN ARE SHOWN FOR EACH CHEMICAL CONGENER AS WELL AS MEDIAN AND ARITHMETIC RANGES. FREQUENCY OF OCCURRENCE (FO) IS SHOWN IN PERCENT. CONCENTRATIONS FOR PCB, PBDE SHOWN AS µG/KG AND DEHP IS SHOWN AS µG/G.DRY MATERIAL. DETECTION LIMITS: PCB = 0.02µG/KG, PBDE 28,47, 99, 100 = 0.02 µG/KG; PBDE 153,154, 183 = 0.5 µG/KG.

Chemical/ Congener	Mean	SEM	Median	Range	FO%
PCB 28	0.3792	0.1009	0.3006	1.01952	100
PCB 52	0.006248	0.006248	0	0.05623	11.1
PCB 101	0.02475	0.01162	0	0.08235	44.4
PCB 118	0.2029	0.09277	0.09138	0.8585	77.8
PCB 138	0.0575	0.0264	0.02964	0.2245	55.6
PCB 153	0.142	0.05861	0.05173	0.48661	100
PCB 180	0.1994	0.07495	0.1024	0.70432	100
ΣPCB	1.012	0.2072	0.7092	1.7881	-
PBDE 28	0.01442	0.01021	0	0.08673	22.2
PBDE 47	0.3661	0.05841	0.348	0.47811	100
PBDE 99	0.06014	0.01467	0.04752	0.146	88.9
PBDE 100	0.009333	0.006472	0	0.05366	22.2
PBDE 153	0	0	0	0	0
PBDE 154	0	0	0	0	0
PBDE 183	0	0	0	0	0
ΣPBDE	0.45	0.05393	0.4389	0.5333	-

3.3.6 CONCENTRATIONS OF EDS IN CANINE FULL EJACULATE

All pooled samples of canine ejaculate contained concentrations of the seven PCB congeners reporting a FO of 100% for all congeners (Table 3.7). PCB congeners 118 and 153 had concentrations of 15.83µg/kg (SEM ± 4.232) and 13.05 µg/kg (SEM ± 3.185) respectively and were the highest concentrations of the seven congeners.

Four congeners of PBDE were detected (Congeners 28, 47, 99, 100). PBDE 47 was present at the highest concentration (0.0825 / µg kg (SEM ± 0.04469)) followed by PBDE 28 (0.0795 µg kg (SEM ± 0.0307). The two congeners with the highest concentration concentrations also had a FO of 100% with the remaining two congeners reporting concentrations above the level of detection had a FO of 67% representing six of the nine samples tested. Congeners 153, 154, 183 had concentrations below the limits of detection in all samples and therefore a FO of 0%.

TABLE 3-7 CONCENTRATIONS OF ENVIRONMENTAL CHEMICALS IN POOLED CANINE FULL EJACULATE. MEAN VALUES WITH STANDARD ERRORS OF THE MEAN ARE SHOWN FOR EACH CHEMICAL CONGENER AS WELL AS MEDIAN AND ARITHMETIC RANGES. FREQUENCY OF OCCURRENCE (FO) IS SHOWN IN PERCENT. CONCENTRATIONS FOR PCB, PBDE SHOWN AS µG/KG AND DEHP IS SHOWN AS µG/G.DRY MATERIAL. DETECTION LIMITS: PCB = 0.02µG/KG, PBDE 28,47, 99, 100 = 0.02 µG/KG; PBDE 153,154, 183 = 0.5 µG/KG.

Chemical/Congener	Mean	SEM	Median	Range	FO%
PCB 28	4.022	1.915	2.572	6.14	100
PCB 52	1.159	0.6541	0.6877	2.114	100
PCB 101	11.53	4.445	9.511	14.994	100
PCB 118	15.83	4.232	16.48	14.621	100
PCB 138	7.523	1.552	7.766	5.357	100
PCB 153	13.05	3.185	13.73	10.969	100
PCB 180	0.3876	0.02321	0.4064	0.0735	100
ΣPCB	53.5	15.71	51.16	54.26	-
PBDE 28	0.07951	0.0307	0.07003	0.10508	100
PBDE 47	0.08251	0.04469	0.0511	0.14495	100
PBDE 99	0.06706	0.03403	0.09053	0.1106	67
PBDE 100	0.03184	0.01664	0.03934	0.05618	67
PBDE 153	0	0	0	0	0
PBDE 154	0	0	0	0	0
PBDE 183	0	0	0	0	0
ΣPBDE	0.2609	0.1144	0.2256	0.39148	-

The congener found to have the highest concentration in prostatic fluid, ejaculate and milk was PBDE 47. This is highlighted in Figure 3.5 in blue. PCB 153 (also highlighted in blue in Figure 3.5) was not the congener with the highest concentration but was detected in all 3 sample types. No one PCB congener showed consistent high concentrations relative to the others in these three fluid media.

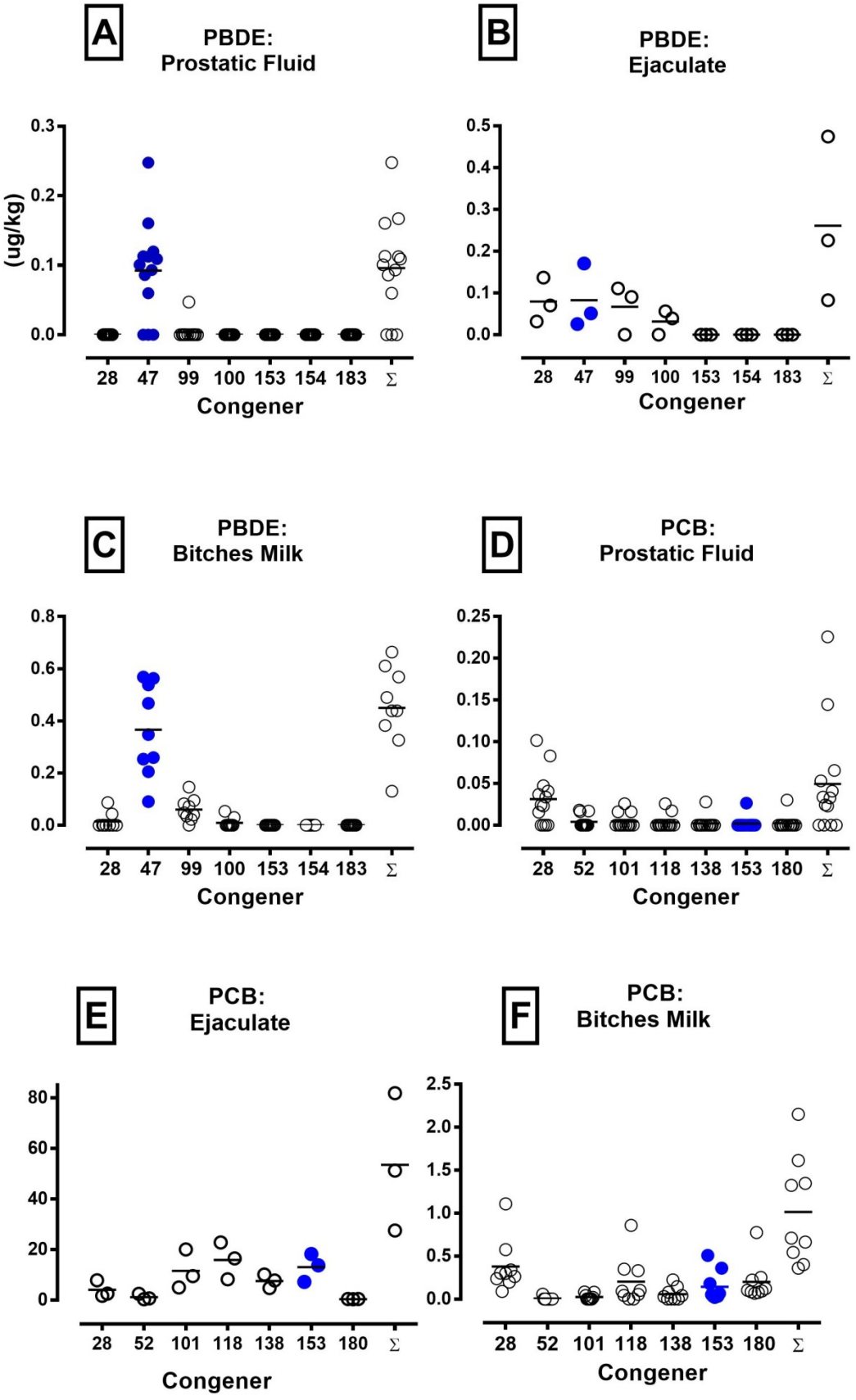


FIGURE 3-5 CONCENTRATIONS OF ENVIRONMENTAL CHEMICALS IN ADULT DOG PROSTATIC FLUID, EJACULATE AND BITCH'S MILK . (A,B,C) PBDE CONGENERS (D,E,F) PCB CONGENERS, MEASURED IN DOG PROSTATIC FLUID, FULL EJACULATE AND BITCH'S MILK RESPECTIVELY. BAR INDICATES MEAN VALUES AND BLUE CIRCLES SHOW CHEMICALS THAT WERE DETECTED WITH GREATER CONSISTENCY ABOVE BASE CONCENTRATIONS IN TESTES AND FOOD. CONCENTRATIONS FOR PCB, PBDE SHOWN AS µG/KG AND DEHP IS SHOWN AS µG/G.DRY MATERIAL.

3.4 DISCUSSION

This study has demonstrated negative temporal trends in specific aspects of canine sperm quality and cryptorchidism in a group of breeding dogs. This is indicative of a change in canine fertility and development in parallel to those reported in humans (Skakkebaek et al., 2001) which has in turn been associated with exposure to environmental pollutants (Bay et al., 2006). Critics of the human TDS hypothesis have questioned methodological and laboratory practices by suggesting that non standardised methodologies create confounding artefacts (Pacey, 2013) in the assessment of human fertility over time. In contrast, in this current study, the temporal data on sperm quality was generated by 3 technicians that were all trained by the same person and all of which used the same established protocol. This suggests that these results are supportive of the human TDS hypothesis and add another dimension to the debate on temporal trends in human, and animal, fertility and reproductive health as it could be argued that this current data is more robust than the human meta data. Additionally, all the dogs in this study cohabitated in a shared environment with their human owners. The relatively short time period, consistent lab practices and a shared environment are all strongly indicative of negative temporal trends parallel to those demonstrated in humans: one parameter indicative of canine TDS with an environmental aetiology. Additionally, a second parameter of TDS has been reported in this current research: cryptorchidism.

The strength of argument suggestive of canine TDS must be put forward with the caveat that other factors beyond sperm quality and cryptorchidism, specifically hypospadias and testis cancer, have not been demonstrated. Hypospadias is rarely reported in the dog (Switonski et al., 2012) and epidemiological data on the incidence of canine testis cancer is limited. There is a suggestion that the incidence rate of canine testis cancer is increasing and histologically similar to human testes cancer (Grieco et al., 2008) supporting a canine TDS. Interestingly,

other reproductive tract disorders like benign prostate hyperplasia (BPH) and prostatitis are commonly seen in dogs (Switonski et al., 2012) and these conditions, in humans, have been linked to oestrogenic pollutant exposures (Shim et al., 2015). These reproductive tract conditions, as they possibly have a common environmental aetiology, may be added to the list of clinical signs of TDS. These, along with the result of our current study, suggest that the canine model of TDS is feasible and warrants further investigation.

An association between cryptorchidism and sex ratios in litters has been suggested (Gubbels et al., 2009). As we have identified a negative trend in the incidence rate of cryptorchidism, it could be hypothesised that over a similar time period there is a possible change in sex ratio of the pups. The GDBA data base of breeding animals lent itself to a straightforward investigation of sex ratio, over a similar time period as the cryptorchidism investigation, to test this hypothesis. The results, shown in Figure 3.2 demonstrate a significant decrease in the number of male pups (litter sex ratio = male pups/total pups) over a similar time period as that used to investigate cryptorchidism (n=21; R^2=0.2; P=0.04). This serendipitous finding suggests a linked mechanism between cryptorchidism and sex ratios as suggested in other studies (Gubbels, et al., 2009). This mechanism although undetermined may be due to perturbed death rates of cryptorchid pups. Interestingly, the change in sex ratio (decrease in male pups) shown herein are in opposition to that previously reported (increase in male pups). This may be due to the GDBA removing cryptorchid dogs from their breeding group, thus selecting against this trait, and the Gubbels (2009) group focusing on carriers for cryptorchidism. Nonetheless, this has practical implications for selection of breeding animals and future research into the mechanisms of both cryptorchidism and sex ratio warrants further investigation. Additionally and perhaps related, an increase in the per cent neonatal survival of male pups in the GDBA population has been identified over the same time period as cryptorchidism. Other researchers have demonstrated in the dog and the pig that the

presence of cryptorchid pups in a litter is linked to an increased male sex ratio (Dolf et al., 2008). This phenomenon may be supportive of our findings. Although intriguing and perhaps associated with cryptorchidism, this phenomenon requires further investigation beyond this preliminary reporting.

Results demonstrate that canine tissue and diet contain EDs. Certain of these EDs were found at concentrations which have been associated with perturbed reproductive development in a sheep model of exposure to an environmentally relevant mixture of chemicals (Lea et al 2014; Lea et al., 2016). Although diet is only one route of exposure to ubiquitous EDs, the result strongly suggest that for certain EDs (PCB 153, PDBE 47 and DEHP) this route needs to be considered due to relatively high concentrations in both diet and tissue. This has implications for the reproductive health of adult dogs but more specifically the canine foetus, exposed through maternal dietary intake to chemical pollutants. Conversely, the results also show that certain EDs (PCB 28, 101) are found at relatively high concentrations in testes tissue but this is not reflected in canine diet as lower concentrations were reported for dog food. This would suggest an additional route of contamination for these EDs, bioaccumulation or both. Overall, the results demonstrate concentrations of certain EDs which have been reported elsewhere as associated with the decline in fertility are found in the canine environment and body.

Exposure to environmental pollutants during foetal, neonatal and pubertal development deserves different consideration. During this time, maternal diet would be one route of contamination but this would precede lactation and weaning. During the neonatal period the pup would be exposed to chemical pollutants via canine milk which has been demonstrated in this chapter to contain detectable concentrations of EDs. Mean concentrations of PCB 153 (0.142µg/kg) reported in this research in canine milk is relatively low when compared to results reported in human breast milk of 12.19 ng/g (Jarrell et al., 2005). An interesting consideration in this comparison is that the women in the work referenced above were

required to be greater than 35 years of age to take part in the study. The dogs age, and therefor opportunity for bioaccumulation of PBCs in adipose tissue, was less and the concentrations reported a reflection of this factor. As a representative congener, PBDE 47 was also found in canine milk at relatively lower concentrations compared to those reports in humans. Human breast milk was found to have PBDE 47 mean concentrations from 3.5 ng/g lipid (Abdallah & Harrad, 2014) to 46.63. Concentrations of Σ7PBDE are reported as 82.242 ng/g lipid (Ryan & Rawn, 2014). Canine milk contained a mean concentrations of PBDE 47 0.366 ug/kg and Σ7PBDE of 0.45µg/kg. The relatively low concentrations of PBDEs in canine milk may be reassuring but possibly a reflection of differences in longevity rather than lower environmental exposures.

Although the differences in PCB and PBDE reported in samples of canine and human milk could be due to different environmental exposures between these species, it could also be due to bioaccumulation during the longer human lifespan of the human. This does suggest that exposures to pups through the lactation may pose a lesser developmental threat than that posed to babies. Another consideration for the human neonate beyond the higher concentrations of PCB and PBDE found in breast milk is the longer nursing period which may be up to one year compared to the convention of weaning pups at 7-8 weeks.

Post weaning diet for dogs will consist of a puppy food for one year as recommended by manufacturers. Of the 28 samples tested for EDs (14 dry food and 14 wet food), six of these were marketed as for puppies. Worryingly, these food contained higher concentrations of DEHP which has anti-androgenic properties (Bornehag et al., 2015). Conversely, the ΣPCB and ΣPBDE mean concentrations are lower (ns) for the puppy food as are the concentrations of the indicator congeners, PCB 153 and PBDE 47 (ns).

Although an interesting observation, beyond the demonstration of concentrations of the specific EDs tested for in this research, any firm conclusions must be stated with caution. Although mean concentrations differed as describe, none of these differences were statistically significant between adult and puppy diet. Retesting of further samples would be required to confirm these initial results as only one sample from one batch of each food type was tested. Any difference in concentrations between puppy food and adult food would also have to be confirmed by more extensive testing across further batches and food brands.

Examples of endocrine disruptors have been proven to be present in the dog's environment through diet and bitches milk. These exposures will result in the presence and bioaccumulation of EDs in tissue and other somatic media. We postulate that the presence and concentrations of chemical pollutants in testes and reproductive fluid will be most directly relevant to reproductive health and function. The concentrations of EDs found in prostatic fluid and their frequency of occurrence are relatively lower when compared to than those found in tissue and food. This may be due to the chemical testing methodologies, as this requires a minimal dry matter which is difficult to achieve with small volumes of fluid which can be collected from dogs with a dry matter of < 1%. Most EDs are lipophilic (Guzmán & Zambrano, 2007) and not hydrosoluble therefore may not be found in high concentrations in prostatic fluid. Further testing is required but other media (blood, adipose) may be better indicators of somatic burden of EDs.

Perhaps more directly related to reproductive function and spermatozoal viability, are concentrations of EDs in full ejaculate. The work of Kamarianos et al. (2003) reported concentrations, across four species, of the sum of five PCB congeners. These researchers showed much lower relative concentrations to those found in the full ejaculate of the dogs tested in our research. We reported the sum of seven PCB congeners in the dog as 53.3 µg/kg which was higher than the highest of the four farm animal species as reported by Kamarianos

et al. (goat 1.44 ng/ml). These high concentrations in the dog may have an impact of sperm function and fertility and warrants further investigation. An interesting consideration may also be that the dog has higher concentrations due to exposures in a different environment than farm animal species: one that is more extensively shared with man. Overall this result is of concern as the PCB concentrations found in full ejaculate are relatively high compared to other fluids.

The congener with the highest concentrations in full canine ejaculate was PBDE 47detected in all samples (FO =100). Mean concentrations for PBDE 47 were 13.2 ng/g lipid detected in human semen samples (Liu et al., 2012) which could be described as relatively high when compared to 0.26µg/kg found in canine full ejaculate.

Congeners PBDE 47 and PCB153 have been identified as congeners of interest due to their relatively high concentrations when compared to other congeners tested in both testes tissue and canine food. This proved to be the case across all media tested for PBDE. In canine food, testes, prostatic fluid, bitches milk and full ejaculate, PBDE47 had the highest concentration. Across the congeners of PCB, food samples and testes tissue had relatively high concentrations of PCB153. In full ejaculate, PCB 118 had the highest concentrations and PCB28 was highest in bitch's milk.

These results support the choice of PCB 153 as an exemplar congener for this chemical group. PCB153 is considered by other researchers as a representative congener of this chemical group. This is, as one of the ICRS 7 and also as one of three 'dominant' congeners (Hussain, Gyori, DeCaprio, & Carpenter, 2000). As one of the six congeners recommended for human biomonitoring, PCB153 is also found in relatively high concentrations in human tissue and serum due to its long biological half-life (Schettgen, Alt, Esser, & Kraus, 2015). In spite of not

being the congener with the highest concentration in all medium it is still a representative single congener for biomonitoring and other experimental purposes in the dog.

Conclusion

The negative temporal trends in canine sperm quality along with a parallel increase in cryptorchidism show parallels with human TDS. The time periods for the changes in canine fertility and incidence rate of cryptorchidism also support those suggestive of an environmental, rather than genetic, aetiology. The contemporaneous decline in semen quality and increase in the incidence of cryptorchidism from the same population of dogs provides robust data without the methodological criticisms directed at the meta-analysis data reporting a decrease in human fertility. The shared human and canine environments also suggest common environmental influences leading to these perturbations of fertility and reproductive development.

All media tested contained concentrations of the target EDs demonstrating that these environmental pollutants are present in the dogs environment, particularly diet, and are present in canine tissue and fluid at concentrations demonstrated to perturb physiological functions. What is also demonstrated in these results and in reviewing the literature is that concentrations of chemical pollutants are variable across different individuals and species. In addition to these specific variations, there may be different population concentrations across geographic regions. As the temporal trends and incidence rates of the components of human TDS vary across geographic regions (Crausaz et al., 2008; Serrano et al., 2013; Swan et al., 2002) this may be a result of variable ubiquity of chemical pollutants in the environment.

As a model for the study of environmental pollutants and reproductive function, as related to fertility and other aspects of TDS, the dog has demonstrated useful in itself and supportive of the work done in other species and humans. This model may also give insight into the

variations in chemical pollutant concentrations across geographic regions both intranationally and internationally. This in turn may provide insight into the variability of aspects of TDS across geographic regions thus supporting an environmental aetiology. The next chapter will investigate these variations across geographic regions.

Chapter 4 GEOGRAPHIC VARIATION AND CANINE FERTILITY

4.1 Background and Literature

4.1.1 Geographic Variation in Male Reproductive Health

Male reproductive health and fertility, in addition to showing a downward trend over the past number of decades (Priskorn et al., 2012; Bongaarts, 2015), have also shown geographic variation (Swan et al., 2002; Crausaz et al., 2008; Serrano et al., 2013). The determining factors which have contributed to this variation have been explored but there has yet to be a consensus as to why this phenomenon is occurring. The most credible hypothesis is that this has to do with either lifestyle (Mah & Wittert, 2010; Skakkebaek et al., 2006) or environmental factors (Nordkap et al., 2012; Buck Louis, 2013; Goossens & Tournaye, 2014). If these trends in reproductive health and fertility are variable by geographic region then it suggests that environmental differences contribute in varying degrees to the downward trends seen in male reproductive health.

4.1.2 Spatial Heterogeneity in Semen Quality

The realisation that differences were being reported in male semen quality from specific geographic regions led to the World Health Organisation (WHO) establishing in 1980 standardised methods for collecting, processing and analysing semen samples (WHO, 2010). This early manual along with the subsequent five editions have gone some way to ensure differences in semen parameters are genuine geographic and regional variations rather than down to inter-laboratory practices. In spite of this, any discussion of geographic or regional differences in semen quality must accept that artefacts created through different skill concentrations and laboratory practices could contribute or even create this phenomenon. Accepting of this caveat, many workers have still reported geographic differences in human semen quality.

A number of researchers have reported variable sperm concentrations in different geographic regions across the United States. Table 4.1 shows the variable results reported and the greatest difference between centres show Seattle to New York with a difference of over 80 X 10^6 /ml. All areas reported different mean concentrations. Although these studies are all retrospective with possible variable methodologies, they are suggestive of a geographic difference in semen quality. Interestingly these studies do not report changes in semen quality over the time of the studies.

These reports of geographic variations in semen quality instigated a specific research study designed in recognition of a need for a standardized protocol to investigate geographic variation in semen quality across the US. This work by Swan et al. (2003) focused on four centres (New York, Minnesota, California, Missouri) using a standard protocol for selection, collection and assessment. Using the centre with the lowest mean concentration (Missouri: 58.7 X 10^6/ml) as a reference value, all centres showed significantly different values on mean count, percent motile and total motile. Only the values for percent normal sperm showed no significant difference. Men from New York showed the highest mean concentrations (102.9 X 10^6/ml) in the study posing an interesting comparison with the values reported previously (See Table 4.1). This is strongly suggestive of differences in reproductive health and fertility by geographic region.

TABLE 4-1 RETROSPECTIVE STUDIES SHOWING MEAN SPERM CONCENTRATIONS IN DIFFERENT GEOGRAPHIC REGIONS OF THE UNITED STATES.

Year(s)	Location	Mean	Reference	Other conclusions
1978 -87	Madison, WI	80 X 10^6/ml	(Wittmaack & Shapiro, 1992)	No temporal trend
1972 – 93	Seattle, WA	50 X 10^6/ml	(Paulsen, Berman, & Wang, 1996)	No temporal trend
1990 -91	Santa Clara County, CA	64 X 10^6/ml	(Fisch et al., 1996)	Stress effect
1970 – 1994	Los Angeles, CA	72 X 10^6/ml	(Fisch et al., 1996)	No temporal trend
1970 – 1994	Minneapolis, MN	100.8 X 10^6/ml	(Fisch et al., 1996)	No temporal trend
1970 – 1994	New York, NY	131.5 X 10^6/ml	(Fisch et al., 1996)	No temporal trend

The much reported negative trends seen in semen quality initiated further investigations into regional differences by other researchers. The observed differences in population supported the theory of an environmental aetiology for the negative temporal trends observed. Early work from Europe, led by Jorgensen and Skakkebaek (2001), set out to identify variation between four cities: Copenhagen, Denmark; Paris, France; Edinburg, Scotland, Turku, Finland. Protocols for recruitment, collection, and assessment were standardised between laboratories. It was found that the men from these four European cities had significant differences in certain semen parameters. Men from Finland had the highest mean sperm concentration (105 ± 73 X 10^6 /ml) with men from Denmark reporting the lowest mean concentrations (77 ± 66 X 10^6 /ml). Men from Paris and Edinburg reported very similar results (94 ± 72 X 10^6 /ml and 92 ± 77 X 10^6 /ml respectively)(Jørgensen et al., 2001). Further work from the same team examined Scandanavian men (Denmark, Norway) and men from the Baltic region (Finland, Estonia, Lithuania) (Jørgensen et al., 2006). This work provided further evidence of differences in semen quality across geographic regions and the relatively poor

quality of semen concentration as a reflection of reproductive health of men from Denmark. Again, this work is strongly suggestive of an environmental aetiology.

Other European regions have also shown geographic variation. A group led by Rolland and Le Moa (2012) demonstrated, using a relatively large cohort of men (n = 26 609) an overall decline in semen quality in France. They followed this with an investigation into semen quality across geographic regions in this same country (Le Moal et al., 2014a). This retrospective study examined concentration, total motility and morphology across a number of geographic regions during the period of 1989 - 2005. The temporal trends overall showed a decline in concentration by regions but with differences in this pattern in specific geographic areas. Other parameters also showed significant differences but no clear overall temporal trends. The authors suggest that these regional discrepancies are supportive of the ED hypothesis with environment as a causative factor.

Spatial heterogeneity in semen quality has been investigated in other countries. In Greenland, where environmental contamination in the population is of concern due to traditional Inuit diet (Van Oostdam et al., 1999), semen quality was assessed in four geographic regions across the country (Toft et al., 2004). An east west difference was particularly noted as men living in the east had lower sperm motility and higher concentrations of PCBs and DDE in their semen samples. Men in Greenland also had relatively low mean concentrations (72 x 10^6 /ml) when compared to most American and European concentrations.

A study of Japanese men found small but significant differences in semen values across four urban locations (Iwamoto et al., 2013). This research also suggested that, if the sample used was representative, Japanese men may have poor, sub optimal semen quality. This work supports the previous suggestion of this group that Japanese men have semen quality equal to the lowest concentrations (Denmark) in Europe (Iwamoto et al., 2007). These reports were

the first to examine fertility across land use categorises, i.e. urban and rural, in addition to geographic boundaries.

Temporal trends in human semen quality are difficult to demonstrate for reasons discussed earlier and this has been well-reviewed (Fisch & Braun, 2013). It must be noted that the evidence of regional variations in semen quality is based on credible, specific, carefully designed studies which are generally prospective and compliant with current WHO protocols. This goes some way to overcome the previously criticised retrospective studies suggesting negative temporal trends in semen quality. The credible research demonstrating geographic variation in semen quality strongly suggests an environmental impact on reproductive health and fertility.

4.1.3 Spatial Heterogeneity and Genital Malformation

The evidence of adverse temporal trends in genital malformation, cryptorchidism and hypospadias, is compelling as reviewed in sections 1.4 – 1.5. These developmental perturbations of the male reproductive tract are showing adverse trends in most geographic regions but some reports suggest this may not be a pan global occurrence as one large study (n = 6935) showed no real variation in incidence when compared to previously reported incidence rates back several decades (Berkowitz et al., 1993). Although it could be concluded, this in itself may be suggestive of a geographic variation in the temporal trends and incidence rates. When the two countries of Finland and Denmark are compared, a clear difference in incidence rate of cryptorchidism is reported (Boisen et al., 2004). This research showed a rate of 9.5% and 2.4% for Denmark and Finland respectively and even when adjusted for all confounders a significant geographic difference was reported. This evidence is compelling as the experimental methodology is robust and less open to criticism as is the work on temporal trends in human sperm quality. Related research data suggests that children in Denmark,

although showing no temporal trends, have a significantly different age of puberty to that reported in the USA (Juul et al., 2006).

The negative trends in human male fertility seen in Danish men, especially when compared to neighbouring Finish men, has been attributed at least in part to higher concentrations of certain environmental pollutants (Shen et al., 2008). This demonstration of differences in male reproductive health between two Nordic countries could be taken as evidence of an environmental factor rather than lifestyle or genetic as causative. A further study examined the incidence rate of cryptorchidism for boys born in Lithuania, which sits geographically between Finland and Denmark (Preiksa et al., 2005). The results showed the incidence rate of cryptorchidism for Lithuanian boys (5.7%) was between the Danish (9.0%) and the Finish (2.4%) boys. Interestingly, Latvian men also had significantly higher sperm counts than Danish (Tsarev et al., 2005) men indicating a geographic difference in overall reproductive health.

A strong argument, supported by the research, suggests factors in the environment are affecting male reproductive health, development and fertility to differing degrees in differing environments. In addition to the differences nationally mentioned above, there has also been work that suggests differences between urban and rural land use within the same country as variations across eastern, central and western regions of China in the incidence rate of hypospadias have been identified (Li et al., 2012). It is largely accepted the TDS has an environmental aetiology and as the components of this syndrome are showing negative temporal trends and geographic variation, these are also suggestive of environmental factors. There is increasing evidence that the associative disorders of cryptorchidism, hypospadias, germ cell cancers and poor sperm quality are due to increasing exposures to EDs (Endocrine Society Statement, 2015). It could be concluded that environmental factors, likely to be

specific pollutants found at varying concentrations across time and geography, are perturbing male reproductive development and subsequent fertility.

4.1.4 Land Cover Data as a tool to investigate environmental factors

The research reporting geographic differences in the collective or individual disorders making up TDS are based on national or regional boundaries. There is a small but important body of work using more precise geographical methodologies. Geographic mapping which identifies land use is one such powerful tool for labelling different features of the biosphere (defined as the layer of the earth which supports life by Eduard Seus (1831 – 1914)).

The CORINE Programme has been established to provide standardised environmental data across the EC member states. This standardised geographic system provides a conceptual tool for identification of special units of homogenous, thematic geographic land cover types. The first level has five land use types: Artificial Surfaces, Agricultural Areas, Forest and Semi-natural Forest, Wetlands, Water Bodies. Each of these five headings is subdivided into 15 further headings at level two and 44 headings at level three. Figure 4.1 shows an example of CORINE Land Cover Data for the UK with an insert listing all headings and subheadings.

CORINE land cover information has been used as a tool to investigate environmental pollution. In Belgium CORINE data was interpolated with concentrations of three pollutants to create an air quality map (Janssen et al., 2008). A model to manage river contamination of residual nitrogen from agricultural practice has been developed using spatial distribution mapping with CORINE land categories (Kersebaum et al., 2003). There are a large number of other examples of CORINE land cover data being used to model air pollution, water pollution and urbanisation

effects on environment (Beelen et al., 2013; Campling et al., 2005; De Meij et al., 2015; Huber, Bach, & Frede, 2000; Trusilova & Churkina, 2008).

There is not any research which uses CORINE land cover data to interpolate map information against tissue or serum concentrations of pollution in animals or humans. In spite of this lack of a specific precedent, this methodology is adaptable to investigate environmental factors and reproductive health of individual animals or humans living within the different land use categories. This would provide a very useful tool which allows investigators another level of analysis beyond national or regional geographic boundaries.

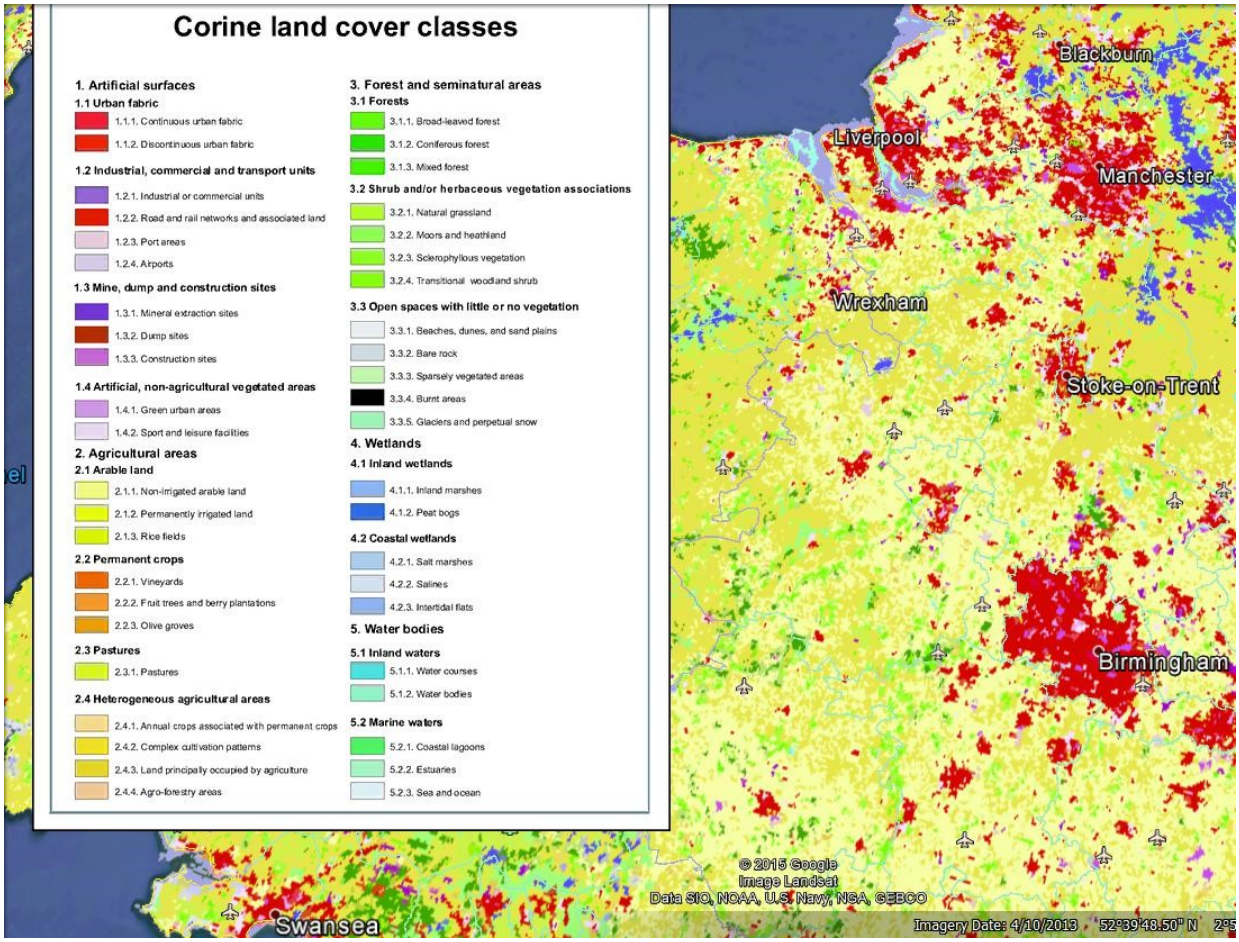


FIGURE 4-1 IMAGE DISPLAYING CORINE LAND USE DATA OVERLAID AGAINST PARTIAL MAP OF ENGLAND. INSERT LISTS MAIN CORINE NOMENCLATURE WITH SUBCATEGORIES. MAP COURTESY OF GOOGLE EARTH (GOOGLE IMAGE LANDSTAT, DATA SIO NOAA, US NAVY NGA GEBCO, CENTRED ON 52° 24 ‘ 32.20’’ N; 2° 31’ 30.73’’ W, ELEVATION 143M, EYE ALT 275.67 KM).

Geographic variation, especially in neighbouring countries, in semen quality and other comorbidity factors of TDS underpin the hypothesis that these ill health end points are the result of exposures to environmental chemicals. An environmental aetiology, along with lifestyle, is a logical explanation for these geographic variations. We hypothesised that differences in chemical profile and/or concentrations found in tissue will reflect differences in exposure to environmental pollutants and that this in turn may account for regional differences in reproductive development and/or function. In order to investigate the above hypothesis, the spatial heterogeneity of chemical profiles and concentrations was investigated in canine testes tissue. This constitutes the main objective of chapter 4 with the following specific aims:

1. Determine and compare chemical profiles in canine testes collected across intra national geographic boundaries.
2. Determine and compare chemical profiles in canine testes collected across international geographic boundaries.
3. Determine and compare chemical profiles in canine testes across different land use types

4.2 METHODS

A full methodology describing the collection of canine tissue samples and chemical analysis is found in Chapter 2.2 – 2.3. The tissue samples were labelled initially, for the purposes of identifying geographic variation, by national boundaries. The UK samples were additionally identified by residential post code data by intra national geographic boundaries, more precisely as either from the East Midlands, West Midland or Southeast.

Based on post code data, residential location of the dogs was categorised based an identification of land use type. A geographic information system (GIS) utilizing Google Earth (Google Inc.) and CLC2006 was used to identify the specific location of the individual dogs by residential post code. The CORINE Land Cover (CLC) is based on satellite images providing standardised and comparable data for the European geographic region. Full detail of the CORINE Land Cover identification is presented in Chapter 2.7.

Statistical analysis was performed using GenStat Discovery Edition 4 statistical analysis package (VSN International) and/or GraphPad Prism 6.07 Windows, GraphPad Software, La Jolla California USA, “www.graphpad.com” as described fully in Chapter 2.14. Means and standard error of the difference values were plotted or expressed in the text and P-values of <0.05 were considered statistically significant.

4.3 RESULTS

The spatial heterogeneity of chemical concentration found in testes tissue of dogs living in three geographic regions of the UK is illustrated in figure 4.2. Strikingly, there was more heterogeneiety in testis chemical concentrations in the West Midlands group. Statistical analyses of each individual chemical revealed a significant difference in ΣPCB congeners (Figure 4.3A: $P<0.05$) but not with respect to ΣPBDE congeners (Fig 4.3B) and DEHP (Fig4.3C) across three UK regions. Concentrations of ΣPCB congeners were higher in the West Midlands (n=9) than in the East Midlands (n = 12) and Southeast (n=14). The area of West Midlands (2.6 µg/kg (SEM 0.64)) showed the highest mean level of ΣPCB with the lowest being the Southeast area (1.1 µg/kg (SEM 0.27)). Although PBDE was not significantly different, Figure 4.2B is indicative of a trend towards higher concentrations in the West Midlands ($P=0.07$). The heterogeneity of the West Midlands cohort was demonstrated by the presence of testes with the highest (2.90µg/kg (SEM 0.60) and lowest concentrations of PBDE (<detection limits). There was no evidence of regional variation in DEHP (Fig 4.3C) although the Southeast did have the highest mean concentrations.

The total chemical burden as represented by the combined concentrations of ΣPCB + ΣPBDE + DEHP showed a significant difference across the three regions with the West Midlands having the highest and most varied distribution of combined concentrations for the three chemical groups ($P<0.05$).

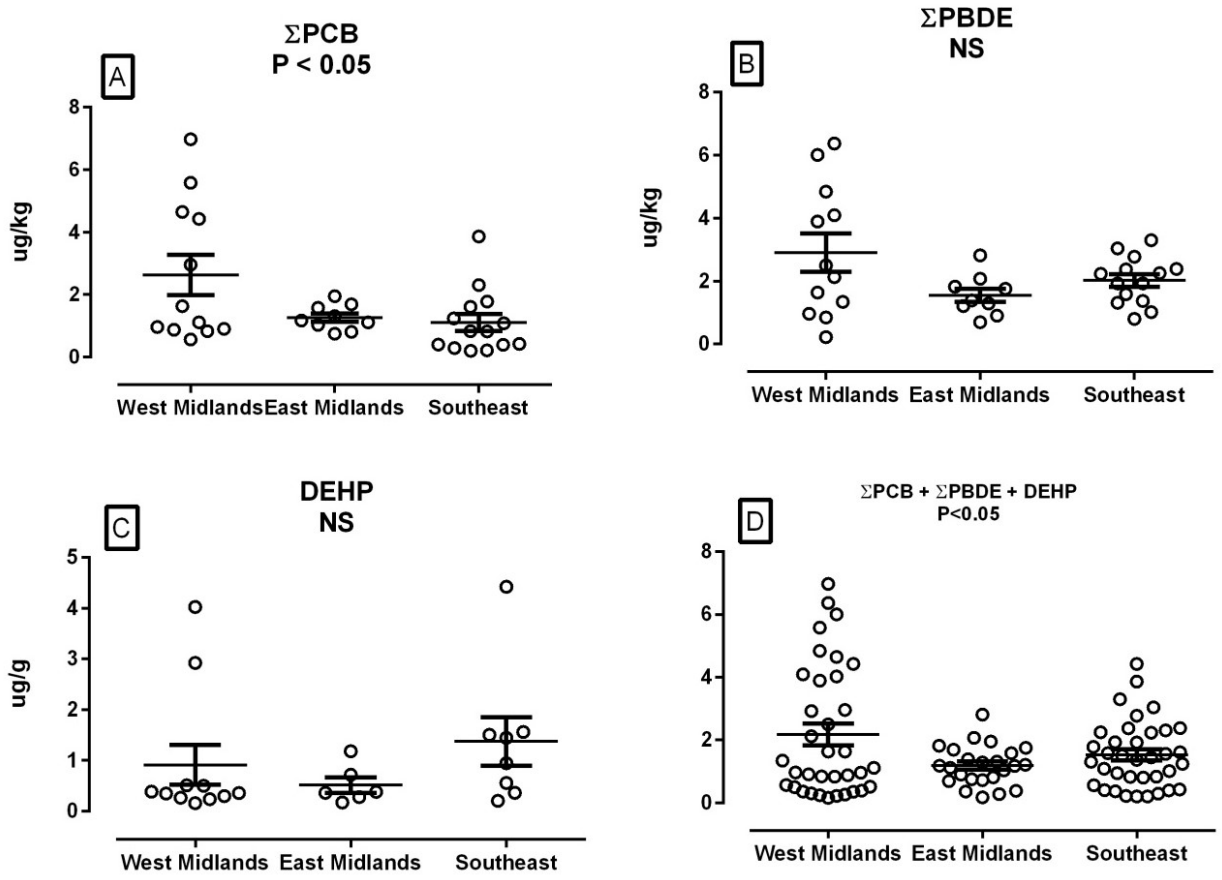


FIGURE 4-2 SPATIAL HETEROGENEITY OF TISSUE CHEMICAL CONCENTRATIONS FOUND IN CANINE TESTES TISSUE ACROSS THREE GEOGRAPHIC REGIONS IN THE UK. THE THREE REGIONS COMPARED WERE THE WEST MIDLANDS, EAST MIDLANDS AND THE SOUTHEAST. (A) REPRESENTS THE MEAN SUM OF SEVEN CONGENERS OF PCB . (B) REPRESENTS THE MEAN SUM OF SEVEN CONGENERS OF PBDE . (C) REPRESENTS MEAN CONCENTRATIONS OF DEHP.. (D) ALLOWS A VISUAL COMPARISON THE MEAN SUM OF ALL THREE CHEMICAL CONCENTRATIONS ACROSS THE THREE REGIONS AS A CONSIDERATION OF TOTAL CHEMICAL BURDEN. CONCENTRATIONS FOR PCB, PBDE ARE SHOWN AS µG/KG AND DEHP IS SHOWN AS µG/G DRY MATERIAL, CONCENTRATIONS FOR TOTALS IN D ARE LEFT IN RESPECTIVE UNITS FOR COMPARISON PURPOSES ONLY. BARS: MEAN ±SEM.

In order to assess the concentrations of individual congeners across the three targeted areas of the UK, individual chemical congeners for PCB and PBDE were examined across the 3 regions (Figure 4.3A - F). PCB153 and PBDE47, identified as congeners of interest, have been highlighted in blue for easy visual comparison. With respect to all three regions, PBDE 47 was present at relatively higher concentrations than the other PBDE congeners (Fig 4.3D, E, F). The three geographic regions also showed a differing chemical profile for PCB. Consistent with data presented in Figure 4.2, identifying the sum of PCB and PBDE congeners, greater variation was observed in the concentrations of a range of PCB congeners and in PBDE 47 in the West

Midlands group. With respect to the PCB congeners, this variation was particularly notable for PCB28, PCB101 and PCB153 (Fig 4.3A vs Fig 4.3B,C). PCB153 was relatively predominant in all 3 regions. What comes from this superficial overview is the warranted investigation of the individual congener PBDE 47 and PCB 153. The concentration of PCB 153 represented 19.10% of the total ΣPCB and PBDE 47 represented 87.66% of the total ΣPBDE.

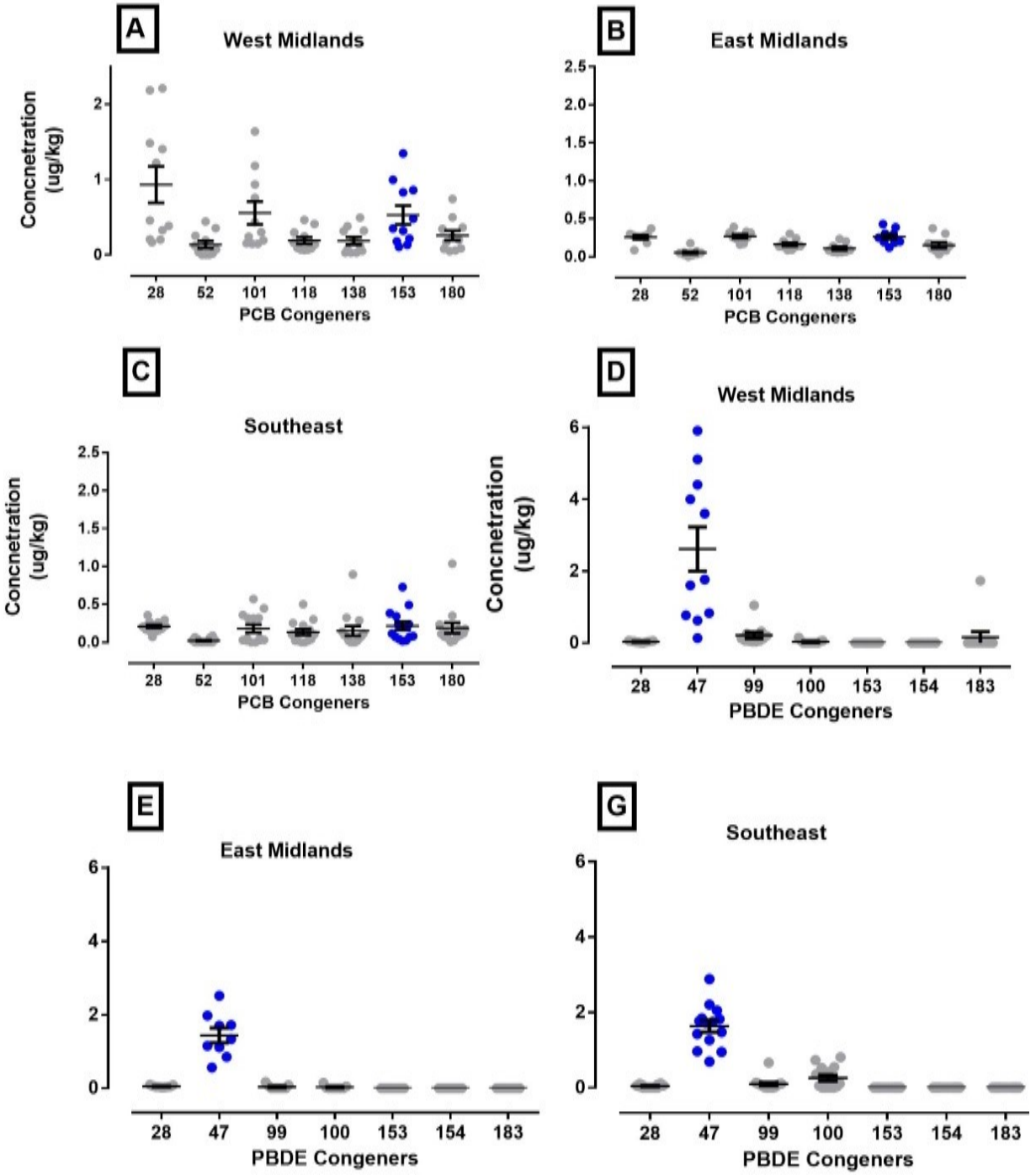


FIGURE 4-3 CHEMICAL PROFILES OF SEVEN CONGENERS OF PCB AND PBDE ACROSS THREE GEOGRAPHIC REGIONS IN THE UK. (A,B,C) ARE THE PROFILE OF CONCENTRATIONS OF THE SEVEN SELECTED PCB CONGENERS IN THE WEST MIDLANDS, EAST MIDLANDS AND SOUTHEAST AREAS OF ENGLAND, (D,E,F) ARE THE PROFILE OF CONCENTRATIONS OF SEVEN SELECTED PBDE CONGENERS IN THE WEST MIDLANDS, EAST MIDLANDS AND SOUTHEAST AREAS OF ENGLAND. DATUM HIGHLIGHTED IN BLUE ARE TO INDICATE CONGENERS OF INTEREST IDENTIFIED PREVIOUSLY DUE TO RELATIVE AND CONSISTENT HIGH CONCENTRATIONS. BARS: MEAN ±SEM.

When examining the individual PCB congener of interest (PCB 153), there was a significant difference ($P < 0.05$) in the concentrations found of this congener across the three geographic regions (Figure 4.4). Again tissue collected from the West Midlands showed the highest (0.52µg/kg, SEM ±0.12) and most diverse concentrations of PCB 153 (Figure 4.4A). The West Midlands region also showed the highest concentration of PBDE 47 (2.61µg/kg (SEM± 0.61), however this was not significant ($P > 0.05$) due to the diversity of concentrations in the West Midlands testes cohort.

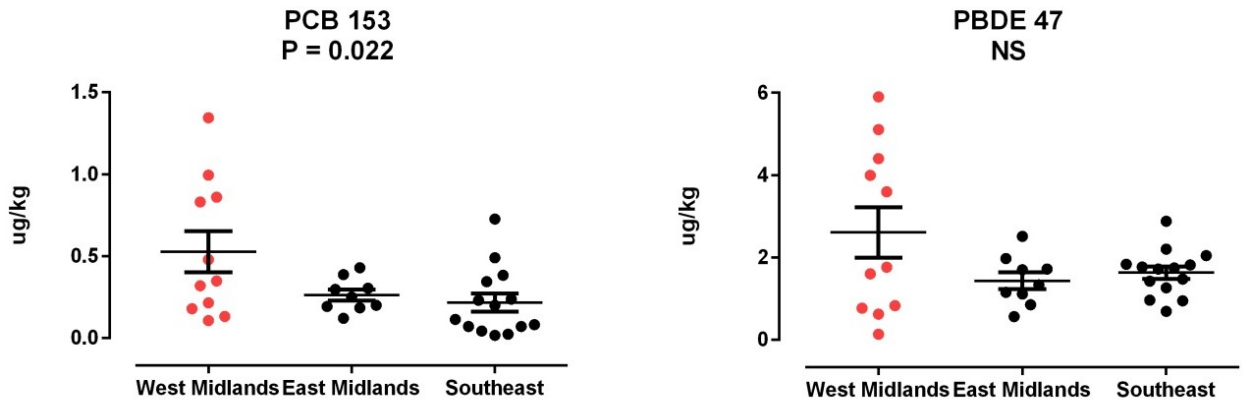


FIGURE 4-4 MEAN CONCENTRATIONS OF PCB 153 AND PBDE 47 ACROSS THREE GEOGRAPHIC REGIONS IN THE UK. THE RED COLOURED DATA POINTS SHOW THE WEST MIDLANDS HIGHLIGHTED AS AN AREA WITH HIGHER MEAN VALUES. THERE IS A SIGNIFICANT DIFFERENCE IN THE MEAN VALUES OF PCB 153 CHEMICALS ($P<0.05$) BUT NO SIGNIFICANT DIFFERENCE IN PBDE47. BARS: MEAN ±SEM.

As age has been determined as a factor in the bio accumulation of PCB and other persistent environmental pollutants the age of the donor dogs was identified per geographic region and shown graphically in figure 4.5. The West Midlands regions had the highest mean age (72.5 months (SEM±11.42) with a significant difference across the three geographic regions ($P<0.05$).

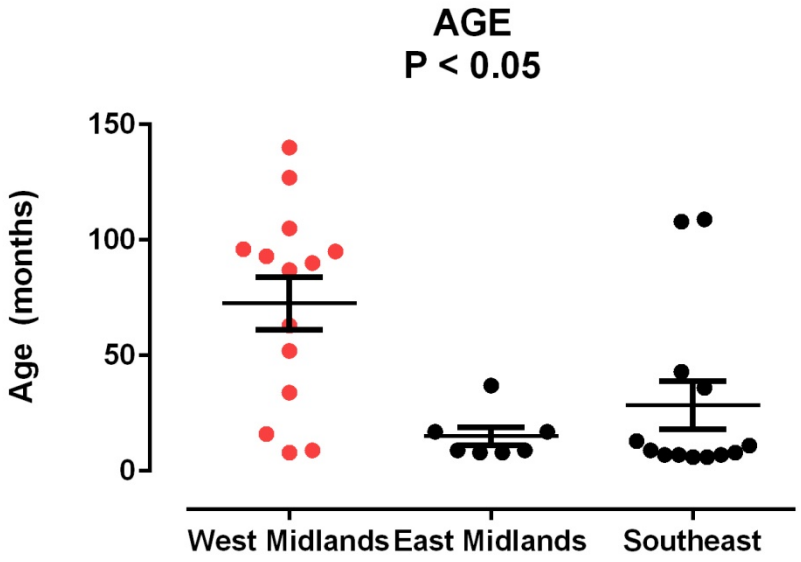


FIGURE 4-5 THE MEAN AGES (± SEM) OF DOGS AT THE TIME OF TISSUE COLLECTION FOR CHEMICAL TESTING FROM THREE GEOGRAPHIC REGIONS OF THE UK: WEST MIDLANDS, EAST MIDLANDS AND SOUTHEAST. THERE WAS A SIGNIFICANT (P < 0.05) DIFFERENCE ACROSS THE REGIONS WITH THE WEST MIDLANDS (HIGHLIGHTED IN RED) HAVING THE HIGHEST MEAN LEVEL.

As there is a significant difference between the ages of the dogs in the three different geographic regions this required further investigation. The correlation between age and the concentration of ΣPCB, ΣPBDE and DEHP is shown in Figure 4.6. There was no significant correlation between age and the tissue concentrations of any of the three chemical groups.

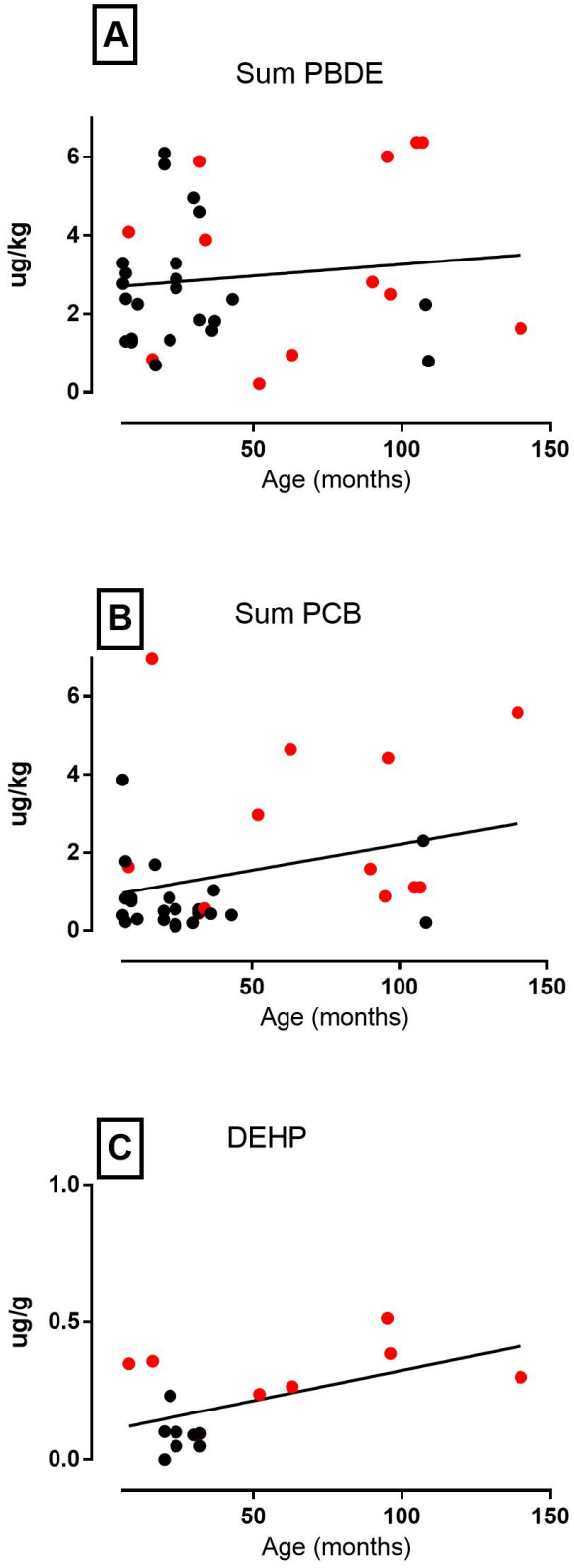


FIGURE 4-6 ASSOCIATION BETWEEN CHEMICAL CONCENTRATION AND AGE FROM TISSUE COLLECTED FROM DOGS IN THE UK. A) SUM PBDE CONCENTRATIONS ILLUSTRATING NO SIGNIFICANT CORRELATION WITH AGE (P > 0.05; R = 0.1248) B) SUM PCB CONCENTRATIONS ILLISTRATING NON SIGNIFICANT CORRELATION WITH AGE (P > 0.05; R = 0.3002) C) DEHP CONCENTRATIONS ILLISTRATING NO SIGNIFICANT CORRELATION WITH AGE (P > 0.05; R = 0.5402). THE DATA POINTS FROM THE WEST MIDLANDS ARE HIGHLIGHTED IN RED. LINE OF BEST FIT SHOWN ONLY FOR DEMONSTRATION NOT AS A REGRESSION ANALYSIS.

Two CORINE land use categories, Agricultural and Artificial, showed a significant difference only in the level of DEHP found in testes tissue from animals sourced in the UK. Figure 4.7C illustrates that dog testes from animals living on the "Artificial Surface" land type had higher concentrations of DEHP (1.42 µg/g (SEM ± 0.33) than those living in Agricultural areas (0.42 µg/g (SEM ± 0.0.07)). In contrast, there were no differences in relation to ΣPCB and ΣPBDE or in relation to total chemical burden.

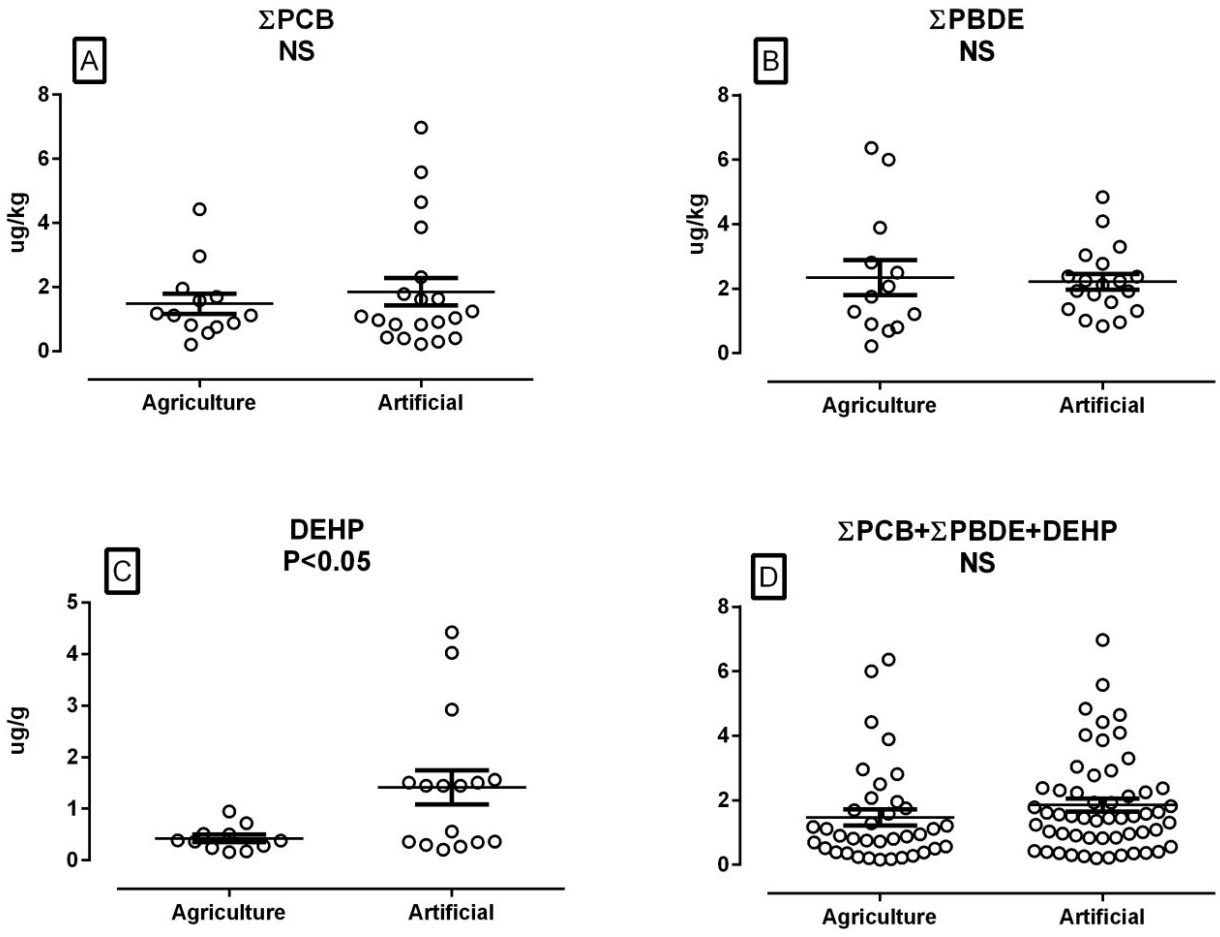


FIGURE 4-7 CONCENTRATIONS OF CHEMICAL CONCENTRATIONS FOUND IN TESTES TISSUE COLLECET IN THE UK AND TWO CORINE LAND USE CATEGORIES (AGRICULTURAL, ARTIFICIAL). A) NO SIGNIFICANT DIFFERENCE (P > 0.05) WAS FOUND IN THE CHEMICAL CONCENTRATIONS OF ΣPCB AND THE TWO CORINE LAND USE CATEGORIES: AGRICULTURE AND ARTIFICIAL. B) NO SIGNIFICANT DIFFERENCE (P > 0.05) WAS FOUND IN THE CHEMICAL CONCENTRATIONS OF ΣPBDE AND THE TWO CORINE LAND USE CATEGORIES: AGRICULTURE AND ARTIFICIAL. C) NO SIGNIFICANT DIFFERENCE (P > 0.05) WAS FOUND IN THE CHEMICAL CONCENTRATIONS OF DEHP AND THE TWO CORINE LAND USE CATEGORIES: AGRICULTURE AND ARTIFICIAL. D) THE COMBINED CONCENTRATIONS OF ALL THREE CHEMICALS REPORTED NO SIGNIFICANT DIFFERENCE BETWEEN THE TWO CORINE LAND USE CATEGORIES. THE UNITS ARE ARBITRARY AND FOR ANALYSIS ONLY .

Chemical profiles of each of the PCB and PBDE congeners are shown in Figure 4.8A-D. The congeners of interest (PCB 153, PBDE 47) show similar, relatively higher concentrations for both of these individual congeners when examined in the two CORINE categories of Artificial and Agricultural land use. For congener PCB 153, this congener represented 15.9% and 21.6% of ΣPCB for Artificial and Agricultural land use types respectively. In both the land use categories, PBDE 47 showed relatively higher concentrations when compared to other congeners. This congener represented 83.7% of the ΣPBDE for Artificial land use and 85.0% of the ΣPBDE for Agricultural land use.

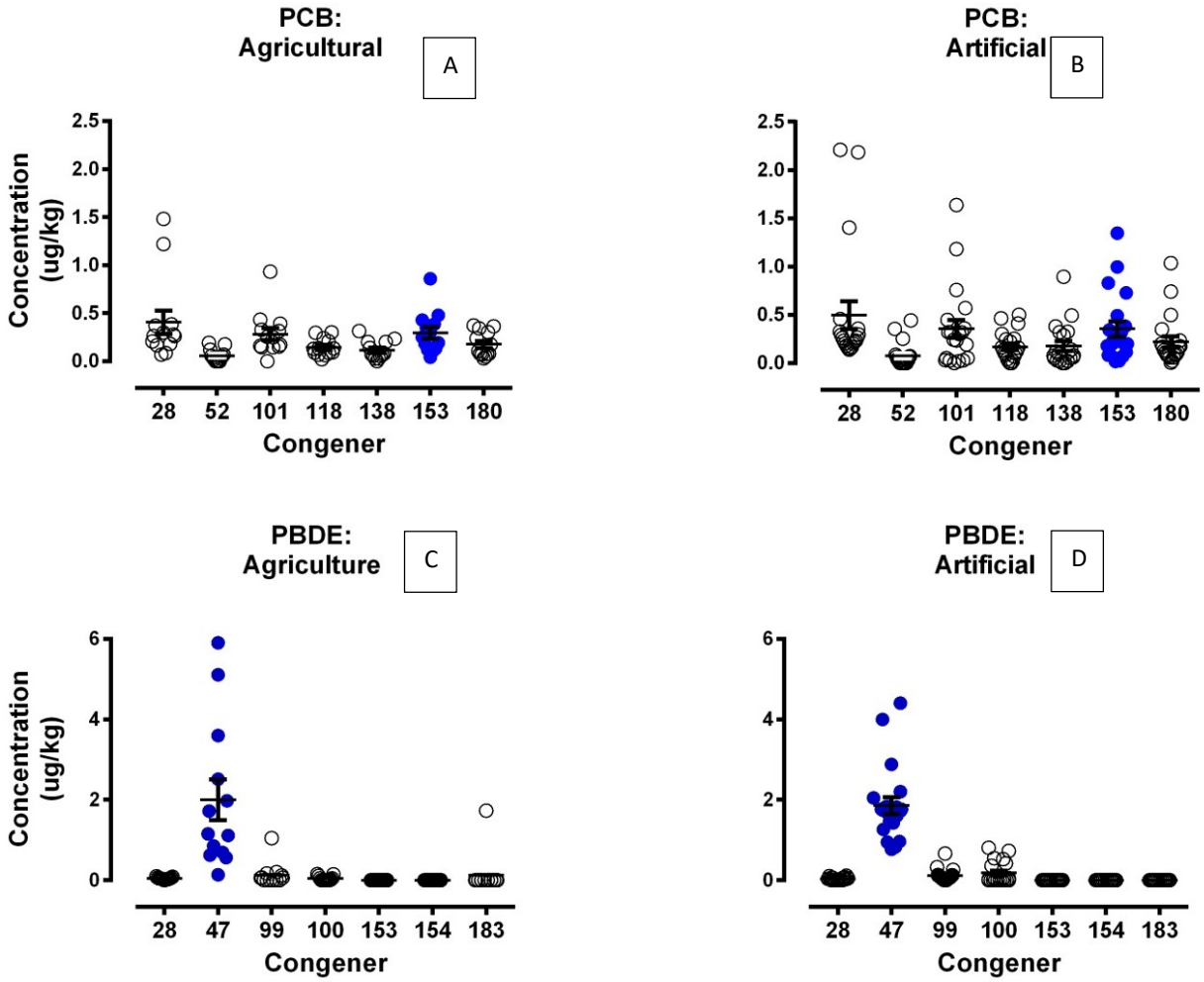


FIGURE 4-8 CHEMICAL PROFILES OF TESTIS PCB AND PBDE CONGENERS IN THE UK BY TWO CORINE LAND USE: ARTIFICIAL AND AGRICULTURE. CONGENERS PCB 153 AND PBDE 47 AS CONGENERS OF SPECIFIC INTEREST ARE HIGHLIGHTER IN BLUE. BAR: MEAN ±SEM.

Individual congeners of PCB 153 and PBDE 47 showed no significant difference between Agricultural and Artificial land use types for tissue samples in the UK region (Figure 4.9).

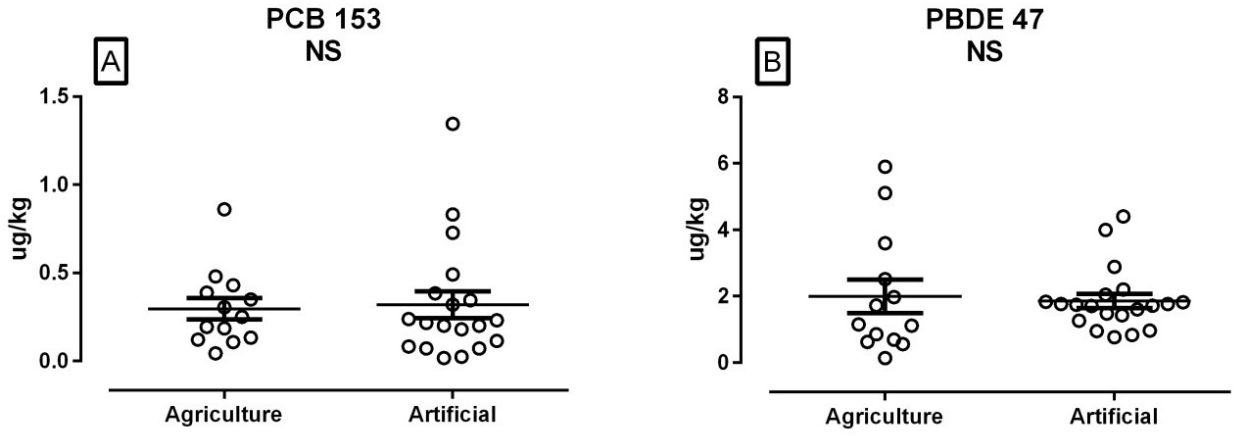


FIGURE 4-9 MEAN CONCENTRATIONS (±SEM) OF CONGENERS 153 AND PBDE 47 FROM TISSUE COLLECTED IN DOGS RESIDING IN THE UK. (A) PCB153, (B) PBDE 47. NOTE THAT THERE WAS NO DIFFERENCE BETWEEN CONCENTRATIONS OF PCB 153 AND PBDE 47 FOR CORINE LAND USE CATEGORIES: AGRICULTURAL AND ARTIFICIAL.

Across international geographic boundaries, there was a significant difference in the concentrations of ΣPCB, ΣPBDE, DEHP between concentrations found in canine testes tissue across samples collected in the UK, Denmark (Copenhagen area) and Finland (Helsinki area) (Figure 4.10). Both mean ΣPCB and DEHP were relatively higher in the UK than the concentrations found in the other two countries. In contrast, the concentrations of ΣPBDE were lowest in the UK. The total chemical burden (Figure 4.10 D) of ΣPCB+ΣPBDE+DEHP showed no significant differences in mean concentrations.

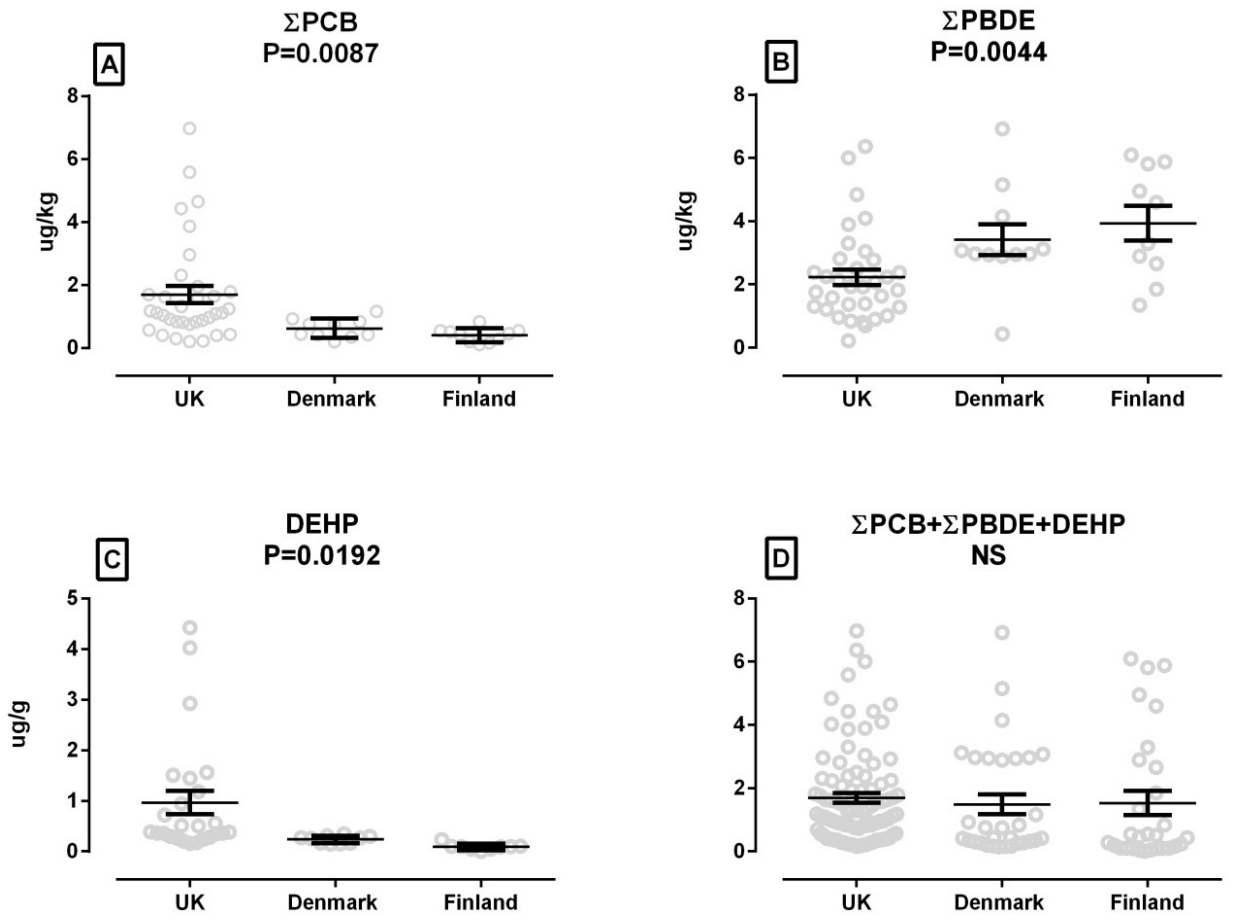


FIGURE 4-10 MEAN TESTIS CONCENTRATIONS OF ΣPCB, ΣPBDE, DEHP ACROSS THREE INTERNATIONAL GEOGRAPHIC BOUNDARIES: UK, DENMARK, FINLAND. (A) MEAN CONCENTRATIONS OF THE SUM OF SEVEN CONGENERS OF PCB (P<0.05) UK HAS THE HIGHEST LEVEL OF ΣPCB. (B) MEAN CONCENTRATIONS OF THE SUM OF SEVEN CONGENERS OF PBDE (P<0.05) UK HAS THE LOWEST LEVEL OF ΣPBDE. (C) MEAN CONCENTRATIONS OF DEHP (P<0.05).. UK HAS THE HIGHEST LEVEL OF DEHP. (D) TOTAL MEAN CONCENTRATIONS OF THREE MEASURED CHEMICALS. THERE IS NO SIGNIFICANT DIFFERENCE IN THESE CONCENTRATIONS.

The individual congener of PBDE 47 showed constantly, relative high concentrations when compared across the three national regions (Figure 4.11D,E,F). This is highlighted in blue for emphasis. The other congener of interest, PCB 153, clearly showed relative high concentrations and greater variability in the UK compared to Finland and Denmark (Figure 4.11A vs B,C). In addition the remaining PCB congeners also showed greater variation in the UK compared to Scandinavia.

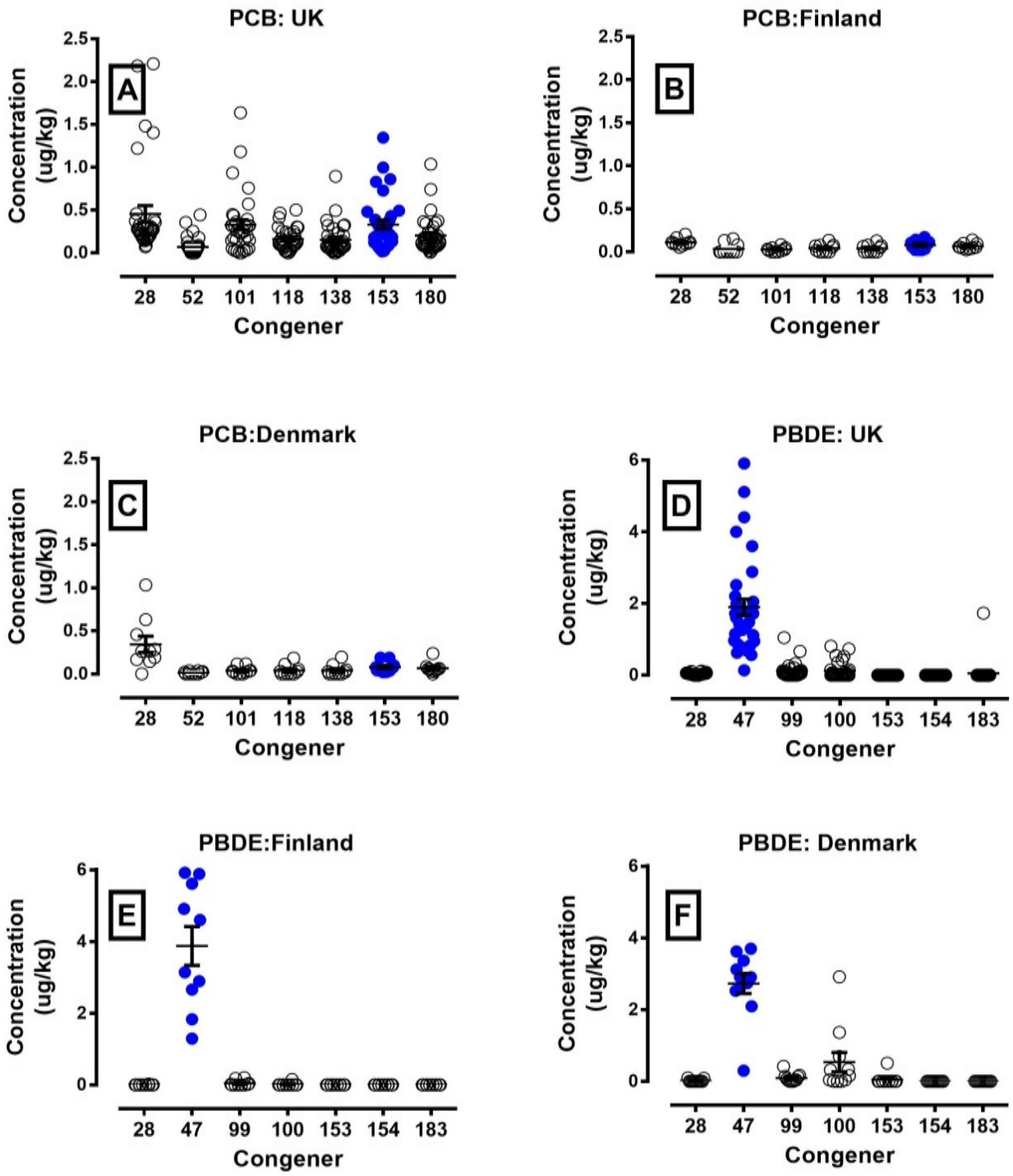


FIGURE 4-11 CHEMICAL PROFILES OF SEVEN CONGENERS OF PCB AND PBDE ACROSS THREE NATIONAL REGIONS: UK, FINLAND, DENMARK. (A,B,C) PCB CONGENERS IN THE UK, FINLAND AND DENMARK (D,E,F) PBDE CONGENERS IN UK, FINLAND AND DENMARK . DATUM HIGHLIGHTED IN BLUE ARE TO INDICATE CONGENERS OF INTEREST IDENTIFIED PREVIOUSLY DUE TO RELATIVE AND CONSISTENT HIGH CONCENTRATIONS. BARS: MEAN ±SEM.

The country with the highest level of PCB 153 was the UK with a mean concentration of 0.23 µg/kg (SEM ± 0.03) compared to 0.08 µg/kg(SEM ± 0.01) and 0.08 µg/kg(SEM ± 0.02) for Denmark and Finland respectively (P<0.01). Alternatively, the concentrations of PBDE 47 were lowest in the UK national region with a mean level of 1.89 µg/kg(SEM ± 0.23) with the other two countries of Denmark (2.72 µg/kg(SEM ± 0.28) and Finland (3.87 µg/kg(SEM ± 0.54) showing relatively higher concentrations (P<0.001).

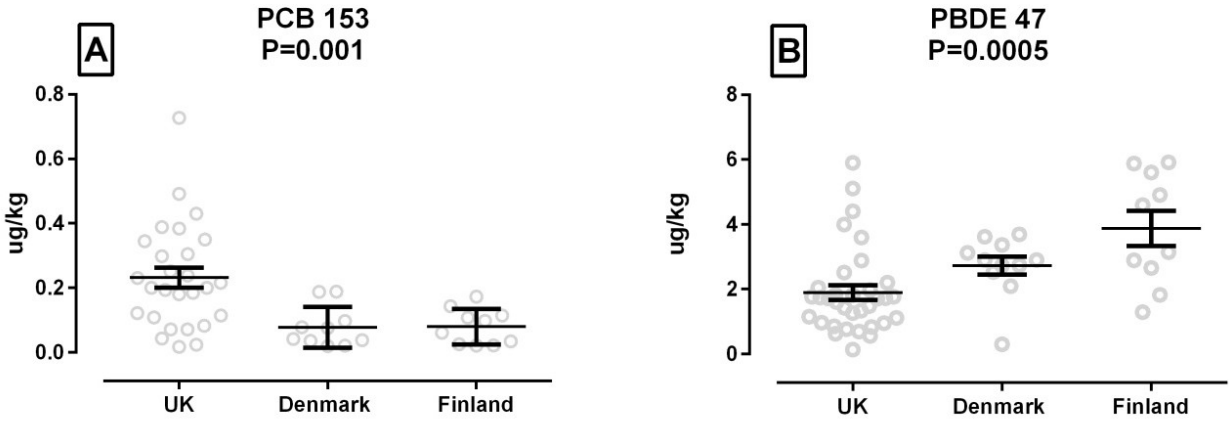


FIGURE 4-12 SPATIAL HETEROGENEITY OF INDIVIDUAL CONGENERS OF INTEREST BY NATIONAL BOUNDARIES. A)MEAN CONCENTRATION (±SEM) FOR CONGENER PCB 153 (P<0.05) B) MEAN CONCENTRATION (±SEM) FOR CONGENER PBDE 47 (P<0.05).

Mean concentrations of ΣPCB for tissue collected in both Agricultural and Artificial CORINE land use types were significantly higher in the UK for Artificial land use areas (Fig 4.13A, P<0.05) but not for Agricultural areas. The concentrations of ΣPBDE across the three countries of UK, Finland and Denmark also showed a significant difference for Artificial land use areas. The UK had the lowest mean level for this compound in CORINE Artificial land use types (Figure 4.13B) with 2.19 µg/kg (SEM ± 0.23). Both Denmark and Finland had higher mean concentrations (respectively: 3.08 µg/kg (SEM ± 0.55); 4.58µg/kg (SEM ± 0.62)).

Figure 4.13 D,E,F shows the concentrations of PCB, PBDE and DEHP across three national geographic regions in the CORINE Agricultural land use type. Statistical analysis could not

determine a difference across these concentrations as the sample size for Denmark (n = 3) and Finland (n = 1) were too low for analysis.

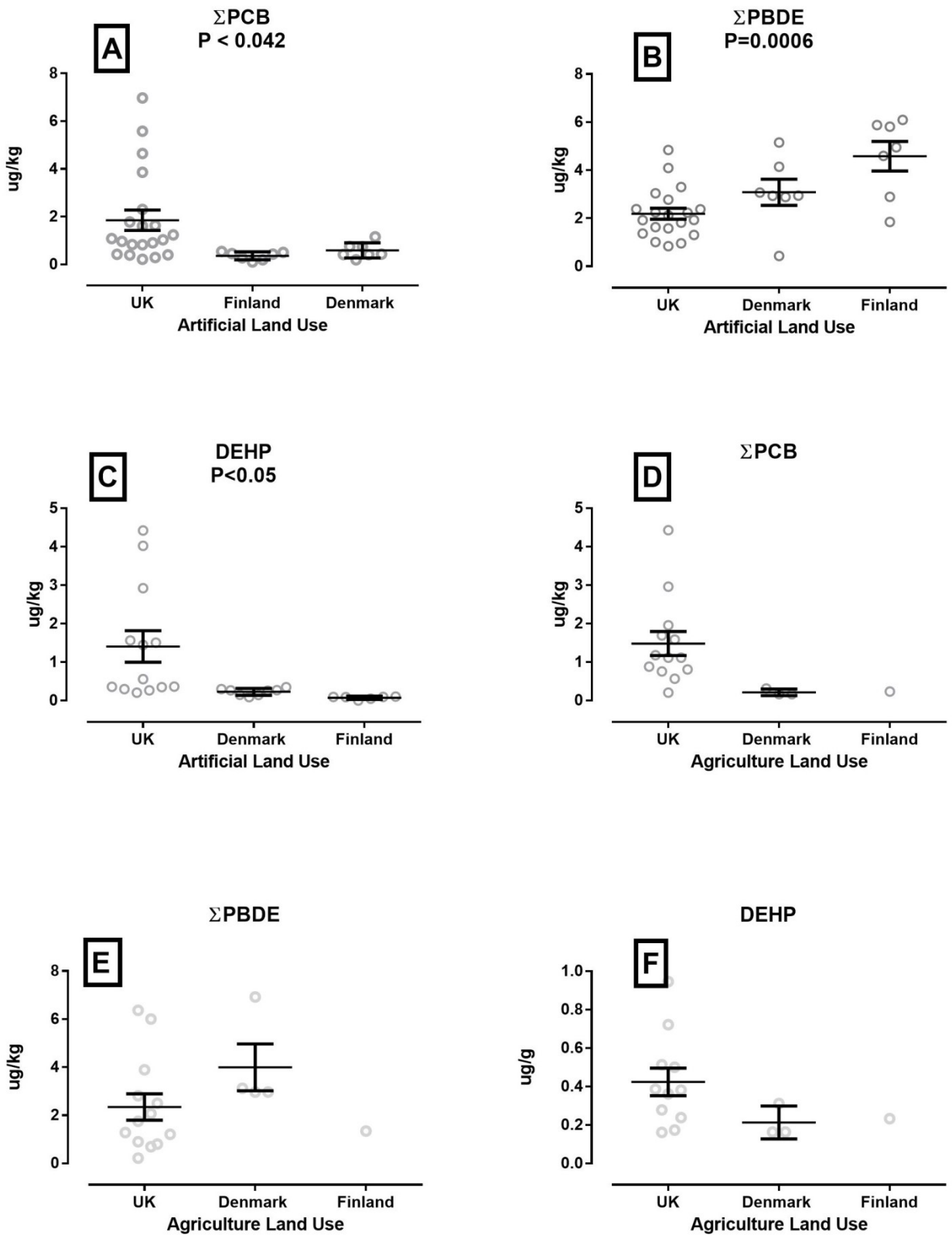


FIGURE 4-13 MEAN CONCENTRATIONS OF ΣPCB, ΣPBDE, DEHP ACROSS THREE COUNTRIES AND TWO DIFFERENT LAND USE TYPES: COUNTRIES: UK, DENMARK, FINLAND; LAND USE: AGRICULTURAL, ARTIFICIAL. (A,B,C): ARTIFICIAL LAND USE, (D,E,F): AGRICULTURE. (A,D) MEAN CONCENTRATIONS OF THE SUM OF SEVEN CONGENERS OF PCB ACROSS UK, DENMARK, FINLAND, ARTIFICIAL LAND USE (P<0.05). (B,E) MEAN CONCENTRATIONS OF THE SUM OF SEVEN CONGENERS OF PBDE ACROSS UK, DENMARK, FINLAND, ARTIFICIAL LAND USE (P<0.05) (C,F) MEAN CONCENTRATIONS OF DEHP ACROSS UK, DENMARK, FINLAND, ARTIFICIAL LAND USE (P<0.05) DIFFERENCE IN THESE CONCENTRATIONS.

4.4 DISCUSSION

Significant differences in concentrations of PCB, PBDE and DEHP were found across intra national geographic regions and international geographic boundaries. This geographic variation in chemical pollutants when considered with reported geographic variation in male reproductive health is intriguing. The fertility and reproductive health of the residents from different regions coupled with these results give further credibility to a proposed environmental aetiology for TDS in humans and possibly in dogs.

In the UK, tissue collected from dogs living in the West Midlands geographic region had the highest concentration of the industrial chemicals PCB and PBDE, both sum of seven congeners and specific congeners of interest (PCB 153, PBDE 47). Therefore it could be suggested that reproductive health of the dogs in these regions may be perturbed to a greater extent than dogs from other regions. This association does not directly indicate causation but does present an area for further investigation. Measurements relating to fertility in these regions should be assessed and compared to the other regions of the UK.

The higher concentrations of the industrial chemical PCB may reflect the industrial legacy of the West Midlands, and the Birmingham area, as well as the current dense consumer based population in the Southeast where the phthalate plasticiser, DEHP, was found at the highest concentration. This speculation must be caveated by the fact that identification of the reason for the differences in chemical concentration by geographic region is beyond the scope of this investigation but intriguing nonetheless. The implications for reproductive development, fertility and reproductive health across these regions would need investigation to fully elucidate the effect of the varying chemical concentrations across geographic variation.

Age of the dogs supplying tissue may be a confounding factor as an association between age and tissue concentrations of chemical pollutants has been suggested in human populations (Schettgen et al., 2015). There is a significant difference between the ages of the sample groups in the different geographic regions with the dogs from the West Midlands area having the oldest mean age of 72.5 months. As there was no significant association between the ages of the dogs from which the tissue was collected and the concentration of ΣPCB, ΣPBDE and DEHP. The conclusion could be that age of the dog does not confound the other results. Therefore other factors, mainly varying environmental pollutant ubiquity, is likely to contribute more fully to the significant differences in the tissue tested from dogs from the three different UK geographic regions.

As there was no significant difference in the concentrations of ΣPCB, ΣPBDE, PCB 153, PBDE 47 when analysed by CORINE land use type or tissue collected in the UK, this does not seem to contribute to overall body concentrations found in the dogs. The movement of the dogs during their lifetime and the residential land use type during foetal development are not considered in these current results but these two factors certainly will contribute to lifetime exposures and therefore overall body concentrations.

The phthalate, DEHP, was found to be at significantly higher concentrations in the land use category *Artificial* for samples collected in the UK. The reason for the phthalate level to be lower in dogs living in Agricultural land use areas would suggest that this chemical had a general lower ubiquity in this area but this cannot be fully determined in this preliminary investigation. It has been reported that PCB concentrations are found in higher concentrations in urban areas (Vorkamp, 2015) but this was not using CORINE roster land use categories but using the researchers determination of rural or urban areas. This type of determination is not repeatable. Our current results are not supportive of these previous

findings based on concentrations found in air samples from cities vs other areas identified as rural.

Across international geographic regions there is a significant difference in the concentration of ΣPCB, ΣPBDE, PCB 153, PBDE 47 and DEHP in the tissue collected. As exposure to these chemicals is via inhalation, absorption or ingestion, these different concentrations found in canine testes tissue would indicate varying concentrations of these compounds in the dog's environments across these different countries or at least from the regions in the countries from which tissue was collected. Aside from some aspect of diet, this could by close association, indicate variable national concentrations of these chemicals in the human environments across these countries. These results are especially interesting when viewed against the spatial heterogeneity reported on both fertility (Jorgensen, 2002) and environmental pollutant concentrations (Shen et al., 2008) found across national boundaries, especially between Finland and Denmark.

When the data is divided by CORINE land use categories (Artificial and Agricultural) the sample size is reduced making statistical analysis of both categories unviable. The statistical difference seen across national boundaries is also evident in the land use category *Artificial*. The sample size for Denmark (n = 3) and Finland (n = 1) for the Agricultural land use category does not allow for credible conclusions. An increased sample size by further tissue collections from these countries would be necessary. This along with a more targeted collection from specific broader geographic areas, fully representative of country, could be an area for ongoing research.

Although the CORINE raster data from 2006 is the most up-to-date available from the European Environmental Data at the time of this analysis the inherent inaccuracies of using historic data must be considered. Also, the boundary issues and locations of residence to

postcode locations are approximations. Although these artefacts need to be recognised they do not negate the overall results.

The chemical profiles showing concentrations of seven congeners PBDE across UK geographic regions as well as all three countries consistently showed congener PBDE 47 at relatively high concentrations. The chemical profiles of the seven PCB congeners indicate that PCB 153 is also relatively high but so also is congener PCB 28. Chemical congeners of interest PBDE47 is the highest in all analyses.

Conclusion

The hypothesis that human TDS has an environmental aetiology linked to EDs has been supported by a number of researchers (Bay et al., 2006; Skakkebaek et al., 2001; Toppari et al., 2010) and regional differences in semen quality and reproductive health have been identified (Jorgensen, 2001; Nordkap et al., 2012; Punab et al., 2002; Swan et al., 2002). These two aspects of human TDS, an environmental aetiology and geographic variation, would suggest that the concentrations of environmental pollutants would vary by geographic region. This chapter presents results which would support this hypothesis. As the closest human companion and an animal which shares the same environment, the chemical burden of the dog should reflect those to which humans are exposed.

The reported geographic variation in concentration of PCB, PBDE and DEHP, known endocrine disruptors, across intra national and international geographic regions is worth more in depth investigation. If environmental factors, which differ geographically, are perturbing reproductive development and subsequent fertility and health this should be reflected in the tissue. A histological assessment of tissue from regions with known differences in chemical concentrations may show associations between these two factors. The following chapter is

designed to assess the histology of canine tissue from these intra and international regions against the known chemical concentrations of the same tissue.

Chapter 5 HISTOMORPHOMETRIC ANALYSIS OF TESTES STRUCTURE AND ASSOCIATIONS BETWEEN CHEMICAL CONCENTRATIONS OF PBCS, PBDES AND DEHP

5.1 INTRODUCTION

The phenotypical development of the testes is initiated by the male genotype but subsequent physiological processes are largely determined by the embryonic and foetal environment. This environment is comprised of maternal and embryonic factors as well as exogenous substances. If these exogenous substances have the ability to perturb normal endocrine function then the development of the testes will be altered. Exposure during the critical programming windows of the testis is associated with TDS and during these developmental periods the testes is most vulnerable (Macleod et al., 2010). If the developmental trajectory is altered there are implications for adult function and ultimately fertility.

Early perturbations of reproductive development are also linked to hypospadias, cryptorchidism and testes cancer. If testicular concentrations of EDs are known, alongside quantifiable histological factors related to reproductive development and function, the relationship between the two can be investigated. Subsequently in the adult, sexual function is controlled by complex systemic, hormonal and neural communications. These communications regulate the action of the hypothalamic pituitary axis. A delicate homeostatic balance maintains normal reproductive function. Working together the hypothalamus and the pituitary glands both receive and transmit chemical signals to the gonads. These sex organs respond in turn to produce both gametes and appropriate sex hormones. This is a simple regulatory mechanism but the overall control of this system is multi-dimensional and includes many systemic and environmental factors. Some of these environmental factors are known to perturb the normal endocrine and gametogenic function of the testes and chemical pollutants are suspected disruptors of these systems.

Since in both the developing embryo and the functioning adult an effect of environmental chemicals may be seen at the cellular level, we hypothesised that measured testis

concentrations of known endocrine disruptors in the dog may be associated with histological indices and maldevelopment. Additionally, if this hypothesis is accepted, testes tissue samples could provide qualitative analysis of both the chemical concentrations and histological scores in order determine a relationship between these measures. This would in turn indicate the effect of specific chemicals on testes development and function.

5.1.1 TESTES DEVELOPMENT AND PERTURBATION

In order to assess the level of insult a polluted environment potentially has on testes structure and function, the process of organogenesis must be fully understood. This understanding will allow for insight into key times during development when the testes are susceptible to negative effects of reproductive toxins and EDs.

A programmed response during gonadal development was firstly demonstrated by Barraclough and Gorski (1961) in well know experiments with pre pubertal rats. These researchers demonstrated that androgen treatment prior to puberty resulted in subsequent infertility in the adult female rat. This was an early indicator of exogenous substances altering reproductive development and led to a classic series of experiments which set the paradigm for developmental programming. This early work was clarified nearly fifty years later by a team of researchers, led by Richard Sharpe, examining the male rodent programme window specifically considering developmental abnormalities i.e. TDS (Welsh et al., 2008). Much was already know about mammalian sex determination, sex phenotype and the bi potential gonad but this work clarified the male programming window and the importance of the hormonal milieu to which the developing foetus is exposed. Again, this work was an indication of the potential for changes in reproductive development of a foetus exposed to environmental or exogenous compounds.

In the human, discovery of allosome chromosomes (X/Y) in 1923 (Painter, 1923) preceded the suggestion of additional factors which determine sex specific phenotype by thirty years. During this period male phenotype was thought to be determined solely by the presence of the Y allosome. This genotypical determinant of male sex was initially identified as a single Y linked gene locus named the Testes Determining Factor (TDF) and subsequently identified as the Sex determining region Y (SRY) gene (Wilhelm et al., 2007). The strongest evidence that the SRY gene initiates the male secondary sexual characteristics and hormonal factors, specifically androgens, contribute to masculinisation is found in Complete Androgen Insensitivity syndrome (CAIS). This important case study in masculinisation, found in one in 20, 000 births, presents as a phenotypical female but has a 46 XY genotype (Hashmi et al., 2008). This is the result of an AR mutation at Xq 11-12 (Oakes et al., 2008) which means the individual, although genetically male, has no functioning testosterone receptors. This is only one of the hundreds of AR mutations identified so far (Gottlieb et al., 2012) but certainly one of the most intriguing when considering male programming and the importance of foetal exposures to hormones. This also demonstrates the importance of factors beyond those related strictly to the genotype and provides interesting insight into the importance of the embryonic and foetal environment. If chemical pollutants have the ability to act antagonistically or agonistically effects on the gonad and sexual characteristics may result. Both of these effects are reported across the congeners of PCB, as an example (Wolff et al., 1997).

The above example demonstrated the difference between genotype and phenotype in the process of reproductive development. The SRY gene begins the process of differentiation but the mammalian gonad is still described as being “bi-potential”. The differentiation of the bi-potential gonad is largely driven by hormonal interactions. This would indicate that the

earliest embryonic stages of development, leading to the pre gonad or genital ridge, would have undifferentiated cellular structure capable of developing towards either sex phenotype. This first early stage sees cellular migration by amoeboid and chemotaxis towards a cluster of cells which creates a foundation of indifferent cells grouped together to form the primordial urogenital ridge which is non sex specificity. As these paired structures comprising the mass of cells destined to become the gonad (testis or ovaries respectively) have not yet come under hormonal influence in their development, this first stage of the gonadal development is of peripheral interest when considering environmental perturbations acting via endocrine function. A caveat to this statement would suggest that environmental chemicals may act through other mechanisms including epigenetic effects.

The second phase of embryonic development initiated by the SRY gene is influence by the hormonal secretions of the embryonic environment and therefore subject to environmental compounds which have the ability to mimic hormonal actions. This is a crucial time when considering environmental chemicals which have endocrine disrupting mechanisms. A subset of somatic cells which are destined to become the nurse cells of the testes initiate the expression of the SRY gene in these pre Sertoli cells. The exact mechanisms of this process are not fully understood (Lyle, 2007) by it has been demonstrated in the mouse embryo that the expression of SRY corresponds with SOX-9 which in turn initiates secretion of Anti-Mullërian Hormone (AMH). AMH is one of the first hormonal secretions of the developing testis. This hormone inhibits the development of the Mullërian (paramesonephric) duct and prevents the development of female gonads. Figure 5.1 shows the sex-specific development of the bipotential gonad and the importance of hormonal influences is demonstrated from this point of development onwards and therefore subject to endocrine disrupting chemical pollutants.

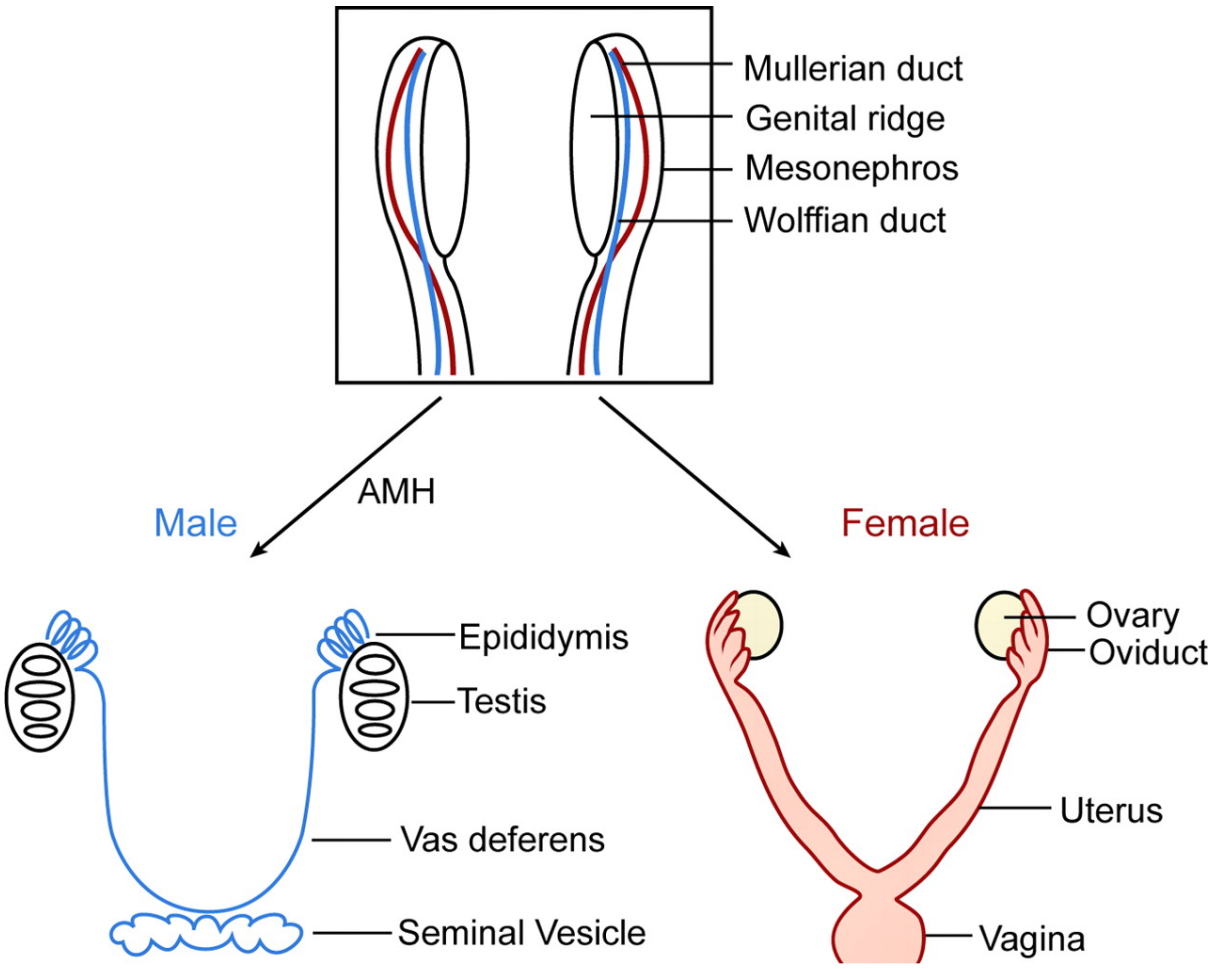


FIGURE 5-1 THE BIPOTENTIAL GONAD WHICH DEMONSTRATES THE IMPORTANCE OF HORMONAL INFLUENCES IN MALE SEX DETERMINATION(WILHELM ET AL., 2007). ANTI MULLËRIAN HORMONE (AMH) INITIATES THE STRUCTURAL DIFFERENTIATION OF THE MALE TESTES.

5.1.2 NORMAL HISTOLOGICAL DEVELOPMENT OF THE TESTES

The migration through the developing embryo of the cells which will populate the testes is occurring on 11.5 – 12.5 days post coitum in the rat testes (O'Shaughnessy & Fowler, 2011). Sertoli cells, colonised into the genital ridge, attract primordial germ cells and epithelial cells. These two cell types migrate via chemotaxis from the yolk sac and the mesonephros respectively due to paracrine growth factors secreted by the Sertoli cells. Once *in situ* within the developing gonad, the differentiation of the Leydig cell development is driven by factors originating in the Sertoli cell (Desert Hedgehog signalling molecule) (Pierucci-Alves, et al. 2001). At this stage (12.5 dpc) Sertoli cell proliferation ceases (Schmahl et al., 2000).

Subsequently, these important gonadal cells are fixed in number during early development and thus the foetal environment can influence adult fertility as Sertoli cell number (i.e testes volume) and sperm production are linked. Any environmental mechanisms which via environmental factors, or otherwise, interfere with or change Sertoli cell proliferation will impact on adult spermatogenesis and therefore fertility.

Post SRY gene and AMH differentiation factors in early development, another critical period of male genital development has been identified and termed 'masculinization programming window' (Welsh et al., 2008). This is an important window when considering environmental chemical pollutants and their effects on reproductive development and subsequent fertility. This next stage in reproductive development begins with the onset of androgen production. In the rat, steroidogenesis begins in developing Leydig cells at 14.5 days post coitum (Hutchison et al., 2008). The Leydig cells, at this point express 3β-hydroxysteroid dehydrogenase which is necessary for androgen production via the Δ5 pathway as shown in Figure 5.2. These events correspond with the structural organisation of the testes (Sharpe, 1989). So again, any chemicals present in the foetal environment which interfere with this steroidogenic process may perturb reproductive development.

During embryonic development, the testis is organised with the proliferation of Sertoli cells, differentiation of the Leydig cells and the peritubular myoid cells (Hutchison et al., 2008). The initial step in this organisation is the cord formation. This occurs at day 12.0 in the mouse embryo (Tilmann & Capel, 1999). Once the testis is formed in its basic structure, it begins as a functioning endocrine gland producing AMH, androgens and Insulin-like 3 (INSL3). The androgens and INSL3 are both Leydig cell productions.

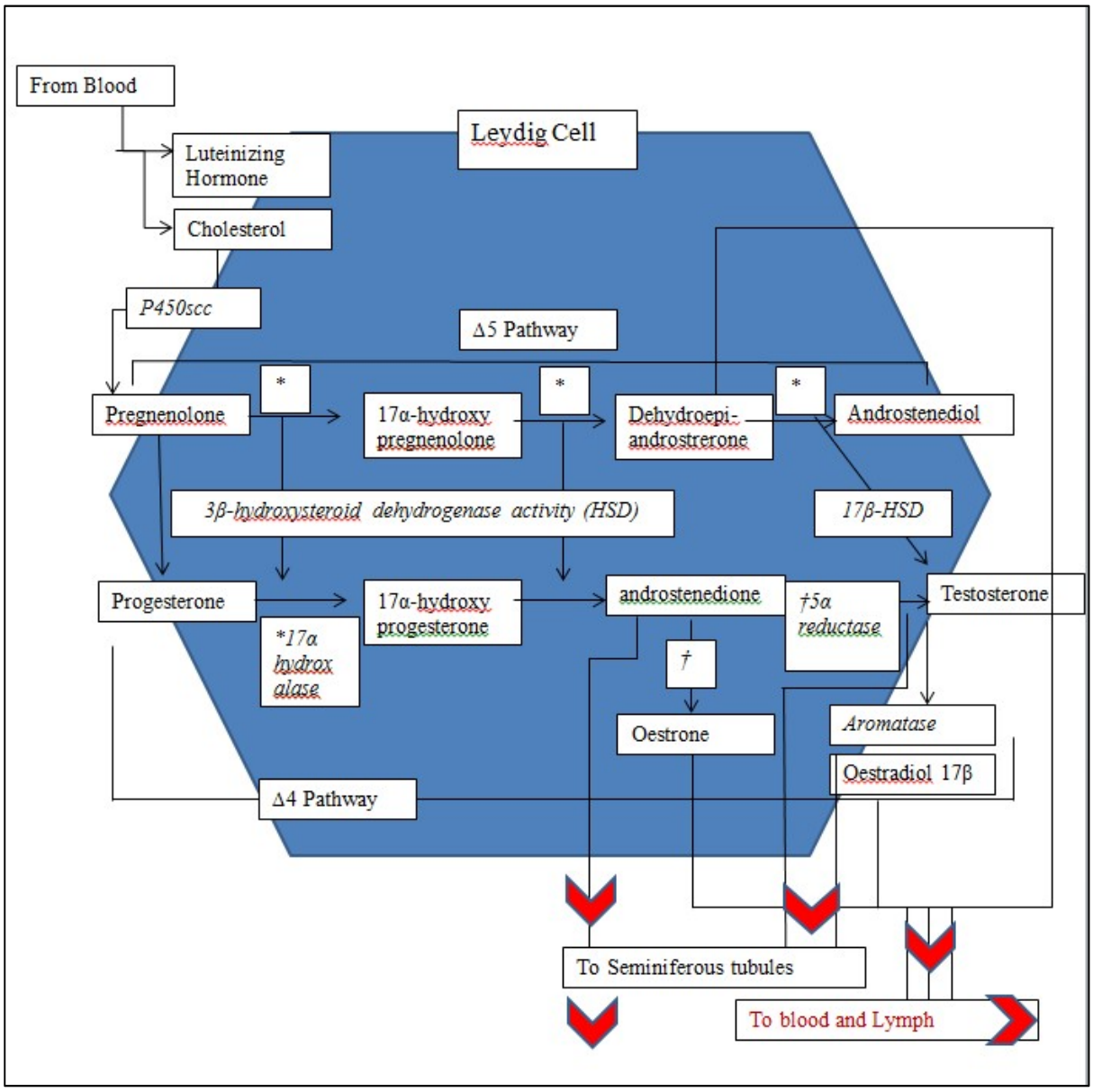


FIGURE 5-2 STEROIDOGENIC PATHWAY IN THE LEYDIG CELL OF THE TESTES. RED ARROWS INDICATE ANDROGEN PRODUCTION MOVING OUT OF THE LEYDIG CELL IN TO THE TUBULES OF THE TESTES AND INTO SYSTEMIC CIRCULATION. ENZYMES ARE INDICATED IN ITALICS.

Early steroidogenesis of the testis must be independent of the developing hypothalamic pituitary gonadal axis (HPG) of adult life. The point at which this axis begins the regulation of gonadal hormone production differs in different mammalian species but a generalised flow diagram (Figure 5.3) shows the basic feedback mechanisms of the HPG axis. Consequently, it is Leydig cell testosterone which is crucial for the 'masculinisation programming window' (O'Shaughnessy & Fowler, 2011). Once the testis is under hormonal influence for development and function it becomes susceptible to perturbations from exogenous hormones

or hormone like substances especially in the early developmental stages. The role generally for these endogenous hormones in the male testis is to masculinise the reproductive organs and subsequently secondary sexual characteristics. This process also diverts the male gonad from the default sex of feminine. The primary hormone in this masculinisation is the testosterone with the first aspect of the masculinisation process to prevent regression of the Wolffian (mesonephric) duct (Schultz & Wilson, 1974;Shaw & Renfree, 2014). The Wolffian duct then forms the ducts of the mature testis including efferent duct, epididymis, ductus deferens.

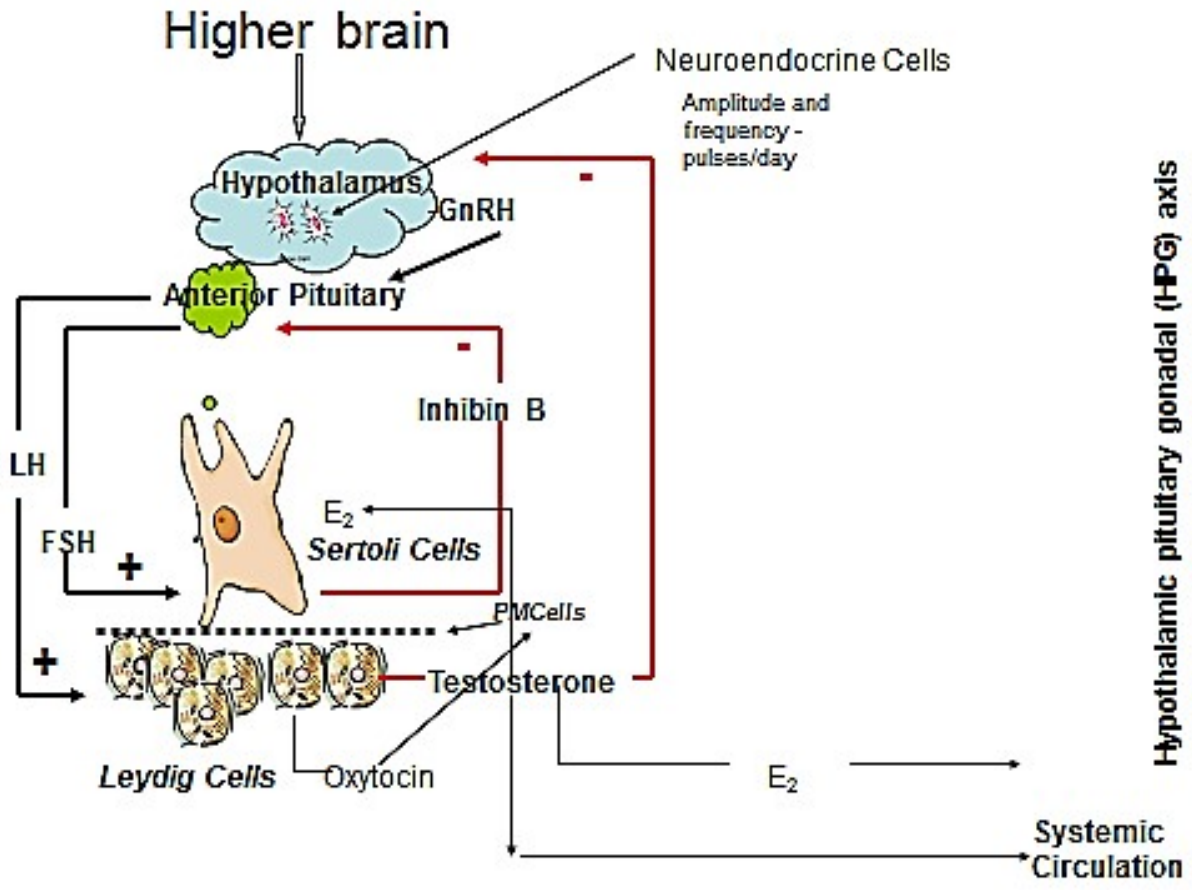


FIGURE 5-3 GENERALISED DIAGRAM SHOWING THE FEEDBACK MECHANISMS AND THE ENDOCRINE GLANDS INVOLVED IN THE HYPOTHALAMIC PITUITARY GONADAL AXIS. THIS DIAGRAM IS NOT SPECIES SPECIFIC BUT REPRESENTS THE TYPICAL MAMMALIAN MODEL.

Systemic testosterone also travels to other sites for the masculinisation of target tissues. This process is enhanced in these tissues by the amplification of testosterone via 5α – reductase. This allows enhanced efficacy of circulating testosterone by conversion to the more potent

dihydrotestosterone (DHT). The testosterone derived DHT androgen hormone is involved in the masculinisation as is testosterone derived oestrogen (via aromatase enzyme)(Sharpe, 2006). One of the organs which is masculinised by androgens is the hypothalamus. This organ is a crucial contributor to the hormonal cascade produced by the HPG axis (Figure 5.7) in its production of Gonadotropin releasing hormone (GnRH). The masculinisation of the hypothalamus is more subtle than the sex dimorphism in other endocrine glands and reproductive organs. The cyclical female function in many mammalian species is altered to an acyclic male gland. This occurs in the ventral medial area of the hypothalamus (Dugger et al., 2007) which contains the Anteroventral, Periventricular and Acuate nuclei (Figure 5.7). The mechanism is not fully known but thought to be due to the larger volume of this area and changes in aromatase activity, both testosterone dependent (Christensen & Gorski, 1978). The important consideration in this process is that if exogenous substances, such as antiandrogenic chemical pollutants, interfere with this process, development and masculinization will be altered. The aromatase activity indicates that testosterone is converted into oestrogen in the hypothalamus as well. These actions are important to comment on as changes to the function of the hypothalamus and subsequent HPG axis will impact on overall endocrine function, especially spermatogenesis.

The process of masculinization of the hypothalamus may not occur during embryonic organogenesis but postnatally in certain species. There is at least a window postnatally when the female rat hypothalamus can be masculinised (Christensen & Gorski, 1978). This oestrogen action, or more specifically oestradial (E2), is a paracrine effect and not due to circulating concentrations of the female hormone but of site specific conversion to this female hormone. This is worth noting, as masculinisation can occur due to the presence of E2 but the substrate is still testosterone. This is demonstrated in Figure 5.2 occurring in the testis but

circulating testosterone is also converted locally in other organs. The presence of oestrogen receptors in many foetal reproductive tissues indicates the importance of this hormone but the full roll in masculinisation is yet to be elucidated (Sharpe, 2006). Thus even this process of masculinisation may be subject to environmental pollutants entering circulation and having oestrogen or antiandrogenic like action (Roselli et al., 2014). There are several pollutant claimed to have such an action including DEHP (Veeramachaneni & Klinefelter, 2013), PCBs (Rignell-Hydbom et al., 2004) and PBDEs (Gray et al., 2006). Although there is evidence that the foetal gonad has the ability to down regulate the testosterone pathway in the presence of exogenous testosterone (Connolly, 2014) thus ameliorating there overall effect.

The development of the penis and descent of the testes are both endocrine dependent masculinisation processes of the normal male gonad and therefore subject to potential negative effects of endocrine disrupting chemicals ubiquitous in the environment. In addition to the male androgens and the female oestrogens, another secretion of the testis is crucial for the developing penis and testes. This hormone, INSL3, is produced in the Leydig cells. Without INSL3 testes descent will not occur resulting in cryptorchidism (Bay & Andersson, 2011) as this hormone initiates the first stage of testes decent via gubernaculum action (Arrighi et al., 2010). The second stage of testes descent is testosterone dependent (Hutson et al., 2013). This is worth noting as INS3 may be altered by EDs during foetal development (Bay & Andersson, 2011) resulting in cryptorchidism: one of the factors in human TDS.

The cytochrome (CYP) P450 super family consist of 57 genes which code for steroidogenic enzymes. The role of the P450 family of genes in the steroidogenic process of the Leydig cell can be seen in Figure 5.2. The specific protein, cytochrome P450 family 11 subfamily A member 1 (CYP11A1) is important in the first stage of steroid synthesis (Shih et al., 2011) and disruption of this gene can cause hormone deficiency (Hu et al., 2002). This is relevant to the

area of research relating to endocrine disruptors remembering that hormonal perturbations during organogenesis and development are suggestive of human TDS. This gene has been used a cell marker by an number of researchers and as an indicator of steroidogenesis in Leydig cells (O'Shaughnessy et al., 2008; Shih et al., 2011; Stanley et al., 2012).

5.1.3 HISTOMORPHOLOGICAL STRUCTURE OF THE TESTES

The structure of the testes is formed early on in embryonic life and the number of Sertoli cells is reported to be linked to testosterone concentrations. Histological examination and quantification of the testes can only be undertaken with knowledge of the cellular structures of the testes. The tissue is organised around 'u' shaped seminiferous tubules which collect, via the tubule lumen, in the efferent ductules leading to the rete testes and ultimately to the epididymis as shown in Figure 5.4. The testis is divided by the ductules into lobes which can be identified in Figure 5.4.

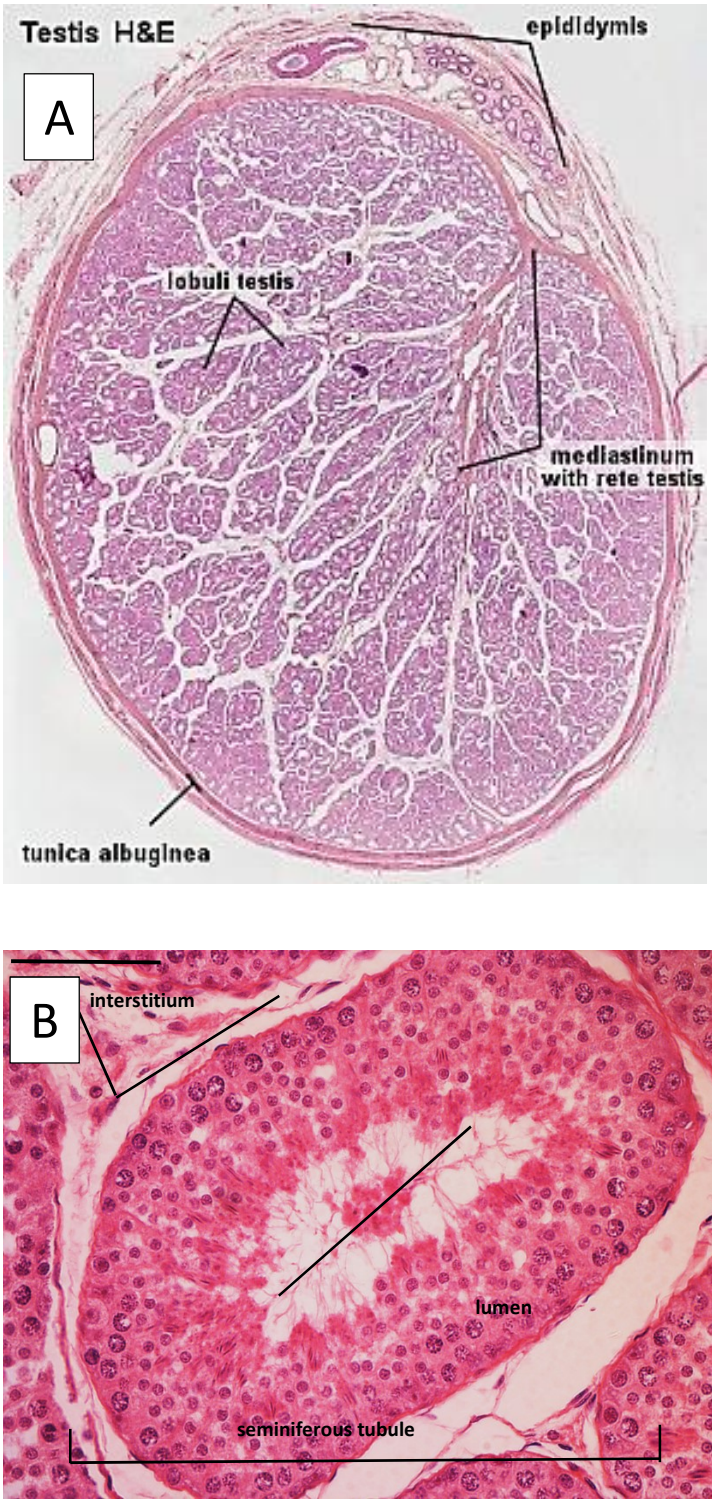


FIGURE 5-4 TESTIS HISTOMORPHOLOGY. A SHOWING ORGANISATION OF GROSS HISTOMORPHOLOGY OF FULL TESTES(40 X) (UNIVERSITY OF WESTERN AUSTRALIA). B IMAGE OF CANINE TESTIS (200X) SHOWING STRUCTURE OF SEMINIFEROUS TUBULE, SCALE BAR = 20 µM.

The volume of testes is made up of three main cell types: Sertoli, Leydig and peritubular myoid cells. The testis is surrounded by a capsule of epithelia tissue referred to as the tunica albuginea testis (Figure 5.5). The capsule is a layered structure containing fibroblasts and

contractile units (Middendorff et al., 2013) as well as blood vessels and smooth muscle cells. This outer layer with its contractile structures provides support and pressure regulation for the testis. The contractile nature may also indicate a function related to movement of fluid and spermatozoa through the testis.



FIGURE 5-5 CAPSULE OF THE MAMMALIAN TESTES. A) CANINE TESTES CAPSULE AT 50X MAGNIFICATION, H&E STAIN. B) CARTOON OF SAME STRUCTURES WITH CLEAR IDENTIFICATION OF CAPSULE STRUCTURES AND LAYERS OF A GENERALISED MAMMALIAN TESTIS (MIDDENDORFF ET AL., 2013).

The interstitial area of the testes, between the seminiferous tubules, contains the Leydig cells and is separate from the tubule by a ring of cells which make up a basal membrane. One of the most important cells of the basal membrane is the peritubular myoid cells (PTM). They play an important role in the intercellular hormonal communication between the Leydig cells and the Sertoli cells and spermatogenesis. This is demonstrated by presence of ARs on the PTM (Welsh et al., 2009) and Sertoli cells but not the germ cells (De Gendt et al., 2004). By definition and as demonstrated by the presence of actin filaments (Maekawa et al., 1991), these cells have a muscular peristalsis like contraction involved in the movement of substances through the lumen of the seminiferous tubule. The number of layers of PTM cells in the basal membrane differs by species, from multi-cell layer found in human testes and a single cell layer in the rat and hamster (Maekawa, Kamimura &Nagano, 1996) with the dog having a tunica propria of 2 – 3 cell layers of PTM (Egger & Witter, 2009).

Within the seminiferous tubule there are only two cell types: the multifunctioning Seroli cell and the germ cells progressing through various stages of spermatogenesis. The germ cells progress through spermatogenesis within the tubule and various stages of this process can be identified by microscopy as seen in Figure 5.6.

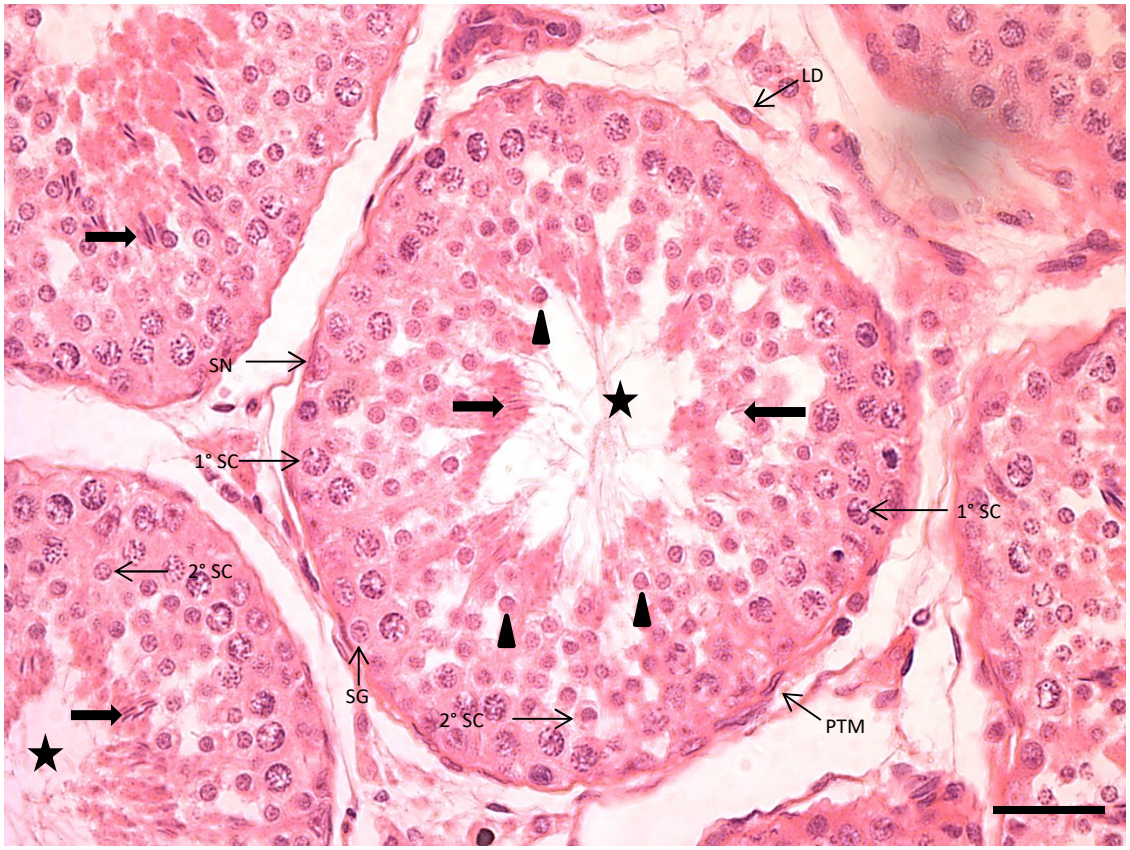


FIGURE 5-6 H&E STAINED, HISTOLOGICAL SECTION OF ADULT CANINE TESTIS (BAR = 20μM). STAR INDICATES LUMEN WITH TAILS OF THE SPERMATIDS PRIOR TO SPERMIATION VISIBLE. ARROW - ELONGATED SPERMATIDS (LATE). LD - INTERSTITIAL LEYDIG CELL. PTM - PERITUBULAR MYOID CELLS. SN - SERTOLI CELL NUCLEUS AT THE BASAL MEMBRANE. TRIANGLE - SPERMATIDS (EARLY). 1° SC - PRIMARY SPERMATOCYTES. 2° SC - SECONDARY SPERMATOCYTES. SG - SPERMATOGONIA.

Quantification of the structures of the testis is necessary when determining a statistical difference between groups based on treatments or other criteria and must be underpinned using the knowledge of the testes outlined above. The majority of these techniques determine structural differences in specific cells or stages of spermatogenesis (Amann, 1981; Berndston, 1977). The methods for gross morphometric evaluation of the testis are less established but this type of assessment has merit when coupled with complementary histomorphological assessments based on cell types or spermatogenesis.

One well recognised method for qualification of spermatogenesis, and therefore testis function, is that put forward by Johnsen (1970). This method scores seminiferous tubules based on the number of cells, at different stages of spermatogenesis (Johnsen, 1970). Although established for and well known as a method for examination of human testes biopsies with over 700 citations in academic literature, this method has been modified for and used in a range of other species; as shown in Table 5.1.

This method of histomorphometric quantification, Johnsen scoring, is an indirect assessment of the cycle of the seminiferous epithelium and therefore spermatogenesis. This cycle is variable in length across different mammalian species lasting from 30 – 75 days (Hess & Renato de Franca, 2008) and approximately 61 days in the dog. The cycle begins with the differentiation of the spermatogonial cells, progressing through divisions and morphological changes and resulting in spermiation which releases elongated spermatids into the seminiferous tubule. These changes in the germ cells are typically grouped into eight stages involving four main cell types: Spermatogonia, Spermatocytes, Spermatids and finally Spermatozoa.

The main cell types listed above are identified when undertaking Johnsen scoring. The presence and quantity of these cells determine the score for an individual seminiferous tubule.

Figure 5.6 demonstrates the cell types found in a canine seminiferous tubule which would be assessed when Johnsen scoring. These cell types relate directly to the four recognised cellular events of the cycle of the seminiferous epithelium. The first stage of this cycle involves proliferation of the spermatogonia, the germ cells which will by mitosis give rise to the second cell type, the spermatocytes. These cells undergo meiosis to form the haploid phenotype of the male gamete developing into an additional cell type called a spermatid. This type of cellular division is also the second sequential event of the seminiferous epithelium. The third cellular event is termed spermiogenesis and involves the maturation of the spermatid. The early stage spermatid, or primary, will be altered in its morphology into what is termed a mature or elongated spermatid. The forth, and final, cellular event releases the mature spermatid into lumen of the seminiferous tubule in a process call spermiation.

TABLE 5-1 EXAMPLES OF THE RANGE OF NON-HUMAN SPECIES WHICH HAVE BEEN STUDIED USING METHODOLOGIES BASED ON JOHNSEN SCORING.

Species	Reference
Rat	(Johnson, Petty, & Neaves, 1980) (Aafjes, Vels, & Schenck, 1980) (Akdogan, Ozguner, Kocak, Oncu, & Cicek, 2004)(Ozguner et al., 2005)(Aral, Karaçal, & Baba, 2008) (Guneli et al., 2008) (Bal et al., 2011) (Esakky, Hansen, Drury, & Moley, 2013)(Tsounapi et al., 2016)(Sönmez et al., 2016)
Mouse	(Lee, Ahn, Jung, Kim, & Lee, 2004)
Rabbit	(Peiper et al., 2009)(Peiper et al., 2006)(Kehinde et al., 2005)
Pig	(Peiper et al., 2006)
Guinea Pig	(W. Kim, Hwang, Lee, Song, & Kim, 2001)
Ferret	(Schoemaker et al., 2008)
Dog	(Peters, de Rooij, Teerds, van Der Gaag, & van Sluijs, 2000)(Larsen, 1977)(Gouletsou, Galatos, Leontides, & Sideri, 2010)
Monkey	(Aravindan, Ravindranath, Gopalakrishnan, & Moudgal, 1990)
Racoon	(Qiang, Murase, & Tsubota, 2003)
Deer	(Pintus, Ros-Santaella, & Garde, 2014)(Pintus, Ros-Santaella, & Garde, 2015)
Bull	(Chapwanya, Callanan, Larkin, Keenan, & Vaughan, 2008)

Strong evidence from epidemiological research in humans suggest an environmental aetiology for decreasing sperm quality and an increase in the incidence rates of cryptorchidism, hypospadias and testis cancer (Bay et al., 2006). Support for this theory comes from variation in measures of reproductive ill health across geographic regions (Jorgensen, 2001; Swan et al., 2002). This is especially poignant when considering culturolinguistically similar regions like Finland and Denmark having differences in sperm quality and other measures of TDS (Jensen, 2000). Specific measure of testes function and structure from tissue collected across geographic regions has not previously been reported but this would further support the theory that environmental factors are effecting testes development and function and therefore fertility. Additionally, land use analysis would support the suggestion that different environments are contributing to this suggested aetiology and this is also a previously unreported assessment.

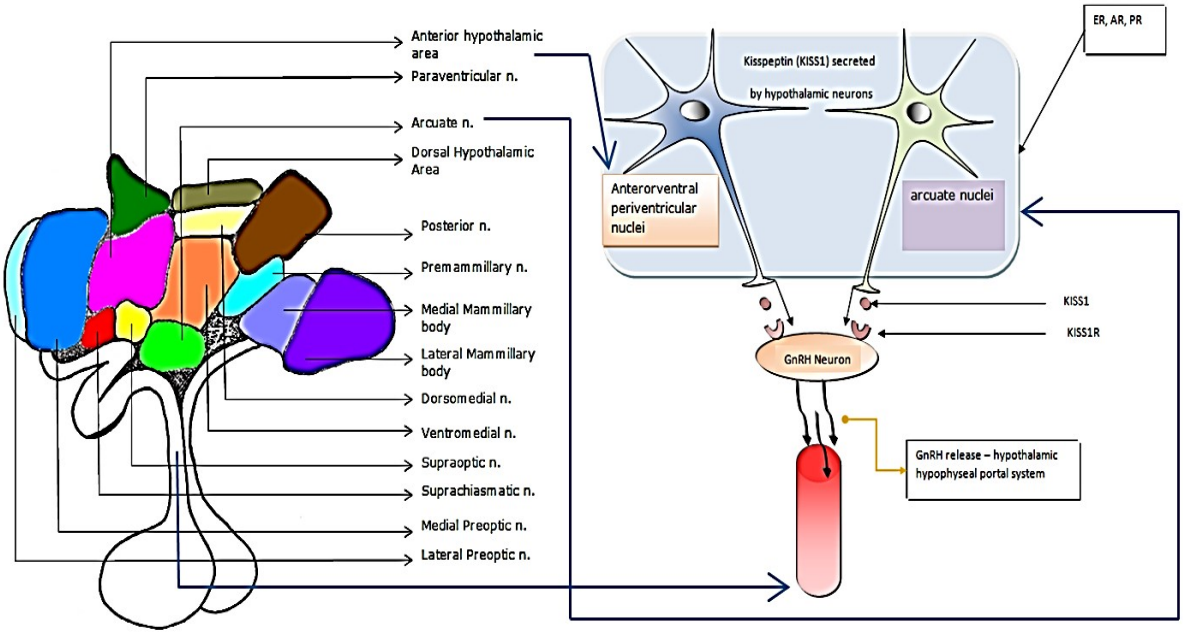


FIGURE 5-7 THE NUCLEI OF THE HYPOTHALAMUS SHOWN IN LEFT HAND ASPECT WITH THE SPECIFIC NUCLEI AND THE SECRETIONS OF GNRH INTO THE HYPOTHALAMIC HYPOPHYSEAL PORTAL SYSTEM AND THE ROLE OF KISSPEPTIN IN THIS HORMONAL CASCADE. ER = OESTROGEN RECEPTOR; AR = ANDROGEN RECEPTOR; PR = PROGESTERONE RECEPTOR.

Gonadal development determines the structure of the post pubertal testes to a large extent and this factor, as supported by endocrine events, is subject to perturbations by environmental chemicals. The function of the testes is regulated by steroidogenesis and hormonal interactions which also may be effected by environmental pollutions. Both of these functional aspects, histological structure and steroidogenesis, can be examined and measured histological. The main aim of this chapter is to determine, using quantitative and semi quantitative methods of histological assessment, if a relationship between testes structures / steroidogenesis can be associated with concentrations of environmental pollutants. In order to determine association between known concentrations of chemical pollutant and testes quality the following objectives are designed to achieve this aim:

1. Examine, using a range of histological methodologies, testes samples with known chemical concentrations for associations between these measures and tissue concentrations of chemical pollutants (PCBs, PBDEs, DEHP).
2. Determine if histological assessments, as determinants of testes structure and function, show correlative relationships and if these differ across geographic regions and land use (in support of an environmental aetiology hypothesis).

5.2 METHODS

Tissue samples were collected, by methodologies described previously, from three national regions. These regions included the countries of UK, Finland (Helsinki area), and Denmark (Copenhagen area). The tissue collected from the UK was divided into three groups representing the geographic regions of East Midlands (n = 7), West Midlands (n = 26) and Southeast (n = 15). The tissue from Finland (n = 10) and Denmark (n = 20) was not sufficient to represent geographic regions beyond Helsinki and Copenhagen respectively. Tissue collection location was, were sample size allowed, examined by CORINE land use categories for differences in measures of testes structure and function across the two categories of Artificial and Agricultural.

Tissue sections were stained with Hematoxylin and Eosin (HE) and were assessed histologically using three scoring methods:

- Gross histomorphology: Tissue sections were examined under bright field microscopy at 40 and 200x magnification. Each tissue section was scored on four important components of testes anatomical structures: organisation of tubules, lumen structure, mediastinum/rete testes, capsule. Full details of assessment criteria are provided in Chapter 2.8.1.
- Johnsen scoring: Based on the method by Johnsen (1970) with modification specific for the canine testis Peters et al. (2000), H&E stained sections were viewed at a magnification of 400x, under standard brightfield microscopy. A total of 100 tubules were scored and the mean was used as the score for the tissue sample as per Johnsen (1970) and Peters et al (2000)

- Tubule size: The diameter through the centre of the tubule lumen was measured for 15 tubules selected randomly per testes using ISCapture software. The mean of the 15 measures was used as a size score for the tissue sample.

A standard immunohistochemical bench method, as describe in Chapter 2.9, was used to stain CYP11A1 as a Leydig cell marker and indicator of steroidogenesis. Primary antibody (rabbit anti-Cytochrome p450 (scc) (CYP11A1) was applied at a concentration of 0.1%. Tissue samples from the UK, Finland and Denmark were collected and prepared for immunostaining using protocols provided in Chapter 2.2 and 2.8.

Staining was achieved by using a DAB substrate for immunoreactivity and for background counter stain, Haematoxylin was used (30 seconds).

Images (40/sample) of the immunohistochemically treated slides were captured using standard brightfield microscopy at 200x total magnification by sequential moving down and over, capturing 10 fields of view starting from each the four poles of the sample. Per cent area stained was determined using image analysis software by quantification of the area of brown stain divided by the area of background stain produced a value for per cent cellular area stained for DAB.

Statistical analysis was performed for associations determined using non parametric Spearman's rank correlation coefficient with GenStat software. ANOVA was conducted using GraphPad software as was unpaired t tests (two way) for examining differences. Means and standard error of the difference values were plotted or expressed in the text and the threshold for statistical significance for P-values was 0.05.

Scoring keys for histomorphology assessment are re-presented for both gross histomorphology and Johnsen scoring in Table 5.2 and 5.3 respectively. Full description of these methodologies is found in Chapter 2.8.1 and 2.8.2.

TABLE 5-2 SCORING CRITERIA FOR GROSS HISTOMORPHOLOGICAL ANALYSIS OF TESTES TISSUE.

Score	Organisation of tubules	Lumen structure	Mediastinum/rete testes	Capsule
0	Pathological, atypical organisation.	No obvious lumen in tubules.	Pathological, atypical.	Pathological, atypical.
1	0 – 49% of testes poorly organised.	Some areas with tubules absent.	Some areas of pathological or atypical mediastinum and or rete testes.	Thin in areas, poorly stratified.
2	Equal areas of disorganisation and typical organisation.	Typical luminal structures.	Almost wholly Typical mediastinum and rete testes structures.	Typical capsular structures
3	Largely organised as typical but still few areas (< 10%) disorganised.	*Limit beyond scale*	*Limit beyond scale*	*Limit beyond scale*
4	Typical, no areas of disorganisation.	*Limit beyond scale*	*Limit beyond scale*	*Limit beyond scale*

TABLE 5-3 CRITERIA FOR TESTES SCORING BASED ON ORIGINAL WORK OF JOHNSEN (1970) AND CANINE SPECIFIC MODIFICATIONS OF PETERS ET AL. (2000).

Score	Johnsen Scoring Criteria
10	Complete spermatogenesis with many spermatozoa. Germinal epithelium organized in a regular thickness leaving an open lumen, or stage 5 of the seminiferous cycle, with sufficient round spermatids.
9	Many spermatozoa present but germinal epithelium disorganized, with marked sloughing or obliteration of the lumen.
8	Only few spermatozoa (< 5–10) present in a tubular cross-section.
7	No spermatozoa but many spermatids present.
6	No spermatozoa and only few spermatids (< 5–10) present.
5	No spermatozoa, no spermatids but several or many spermatocytes present.
4	Only few spermatocytes (< 5) and no spermatids or spermatozoa present.
3	Spermatogonia are the only germ cells present.
2	No germ cells but only Sertoli cells present.
1	No cells in tubular cross-section.

5.3 RESULTS

Samples of testes from the UK showed a significant correlation between histomorphometric scores and chemical concentrations of PCB, PBDE and DEHP. A significant positive correlation was identified between four congeners of PCB (101, 118, 138, 153), PBDE99,DEHP and gross histomorphology scores. Significant correlations for gross histomorphology of UK samples are shown in Table 5.4. The observed relationships between histomorphometric measures and chemical concentrations are graphically displayed in Figure 5.8A - H.

TABLE 5-4 SIGNIFICANT ASSOCIATIONS SHOWN IN THE THREE HISTOLOGICAL SCORING METHODS OF GROSS HISTOMORPHOLOGY SCORE (GS), JOHNSEN SCORE (JS) AND TUBULE SIZE(TS) AND CHEMICAL CONCENTRATIONS REPORTED FOR CANINE TESTES TISSUE COLLECTED IN THE UK.

Assessment	Chemical	P value	r value	N value
GS	PCB 101	< 0.05	= 0.280	= 32
GS	PCB 118	< 0.05	= 0.226	= 32
GS	PCB 138	< 0.05	= 0.227	= 32
GS	PCB 153	< 0.05	= 0.236	= 32
GS	DEHP	< 0.05	= 0.49	= 24
JS	PBDE 99	< 0.001	= 0.235	= 32
HS	PBDE 99	<0.05	= 0.246	= 32
TS	PBDE 99	< 0.05	= 0.394	= 32
TS	ΣPCB	< 0.05	= 0.239	= 32
TS	PCB 101	< 0.05	= 0.235	= 32
TS	DEHP	< 0.05	= 0.299	= 24

One chemical only (PBDE99) showed a significant positive relationship the histological measure Johnsen Scoring (Figure 5.8C). Tubule diameter showed a positive correlation with three chemicals (PBDE99, ΣPCB, PCB101: Figures 5.8B, 5.13A, 5.8D). It is worth noting that

PBDE99 showed a positive correlation with all three histomorphometric scores as shown in Table 5.4 and this relationship is graphically represented in Figure 5.8A, B, C. Also worthy of note is that all significant correlations found with between chemical concentrations and histomorphometric scores for tissues from the UK were positive.

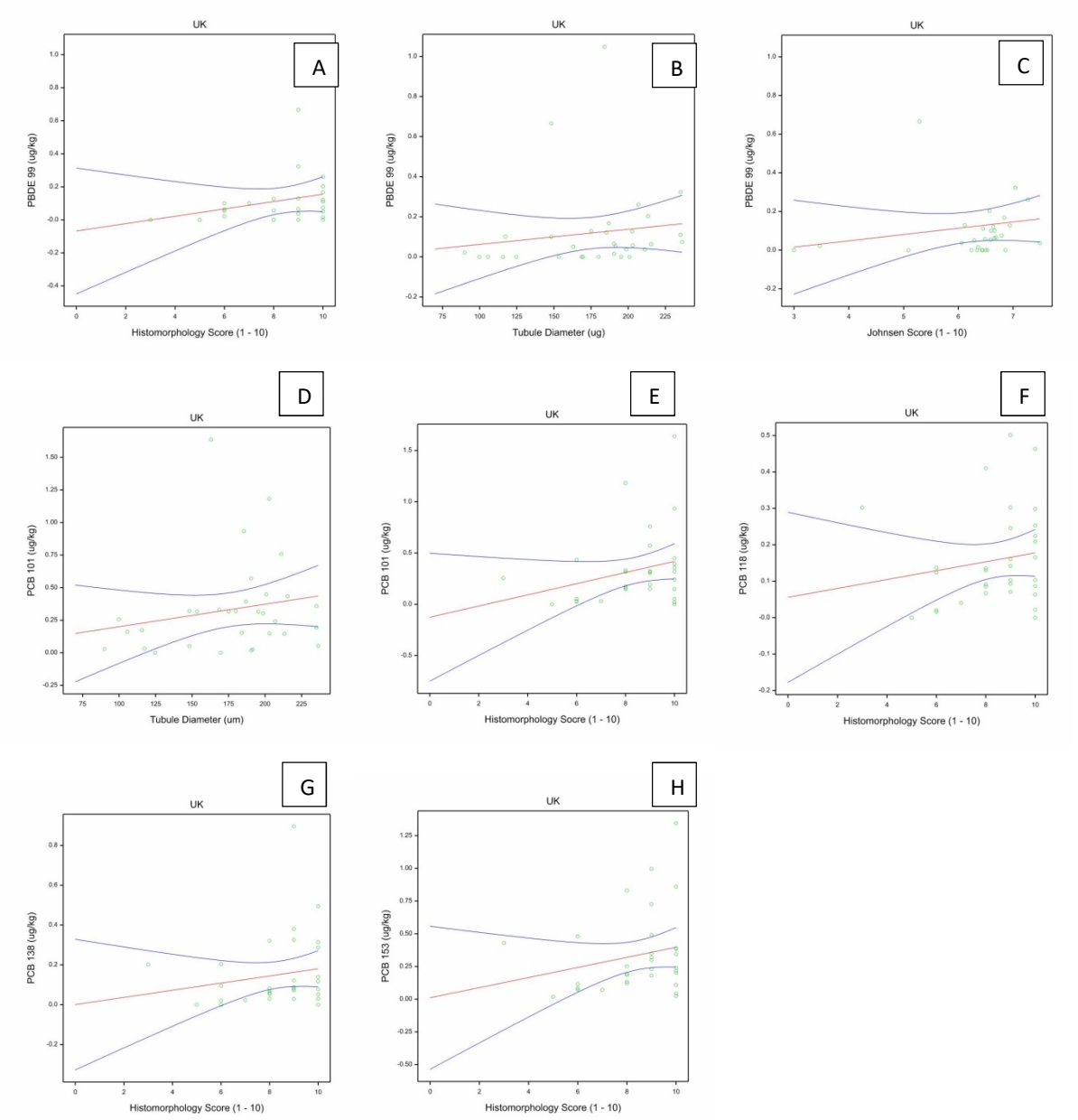


FIGURE 5-8 SIGNIFICANT CORRELATIONS (P < 0.05) BETWEEN PCB & PBDE CONCENTRATIONS AND HISTOMETRIC MEASURES OF TISSUE COLLECTED IN THE UK. LINE REPRESENTS FITTED AND OBSERVED RELATIONSHIP WITH A 95% CONFIDENCE LIMITS SHOWN.

Samples from the three geographic regions in the UK (East Midlands, West Midland, Southeast) showed no significant difference in the gross histomorphological scoring (P > 0.05)

but there was a significant difference in the mean Johnsen score ($P < 0.05$) and the mean tubule size ($P < 0.05$) across the three regions of East Midlands, West Midlands and Southeast of England. These analyses are shown graphically in figure 5.9A - C.

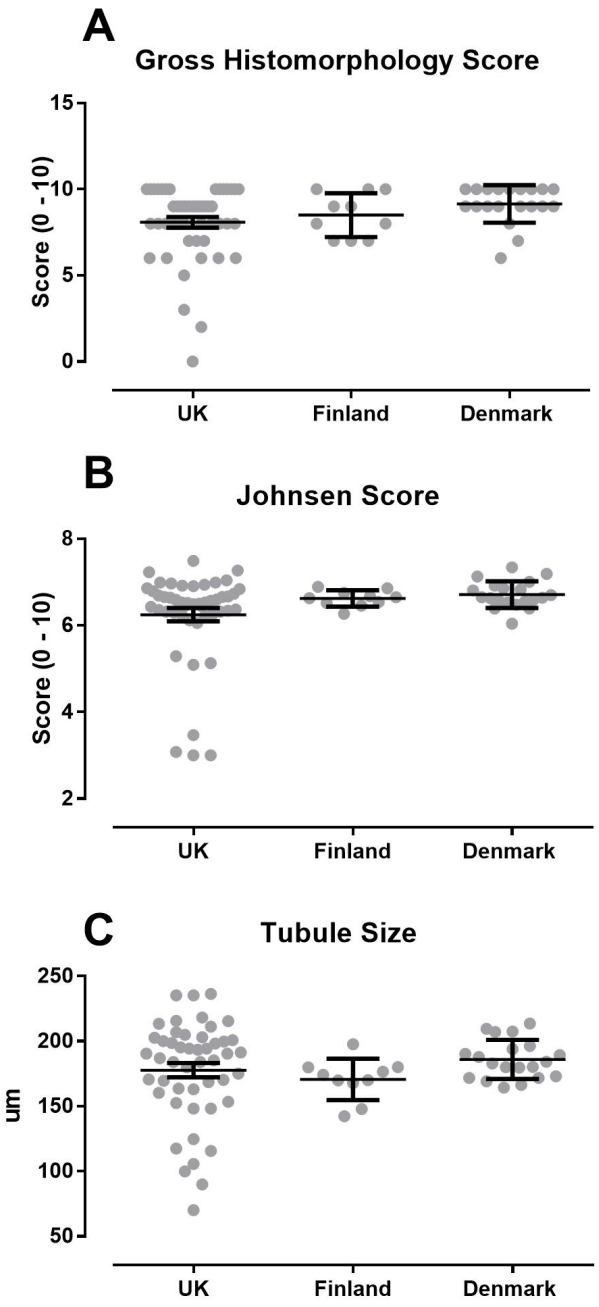


FIGURE 5-9 HISTOLOGICAL ASSESSMENT OF TISSUE SAMPLES COLLECTED IN THE UK ACROSS THREE REGIONS IN ENGLAND. A) MEAN GROSS HISTOMORPHOLOGICAL SCORING OF THE TESTES TISSUE B) MEAN JOHNSEN SCORING OF THE TESTES TISSUE ($P < 0.05$) C) MEAN TUBULE SIZE OF THE TESTES TISSUE SAMPLES COLLECTED ($P<0.0.5$)..

CORINE land use categories, as introduced in a Chapter 4, in the UK were examined against histological scoring and no significant correlations was reported between gross histomorphology score (Figure 5.10A), Johnsen score (Figure 5.10B), tubule size (Figure 5.10C) and CORINE land use categories of Agriculture or Artificial.

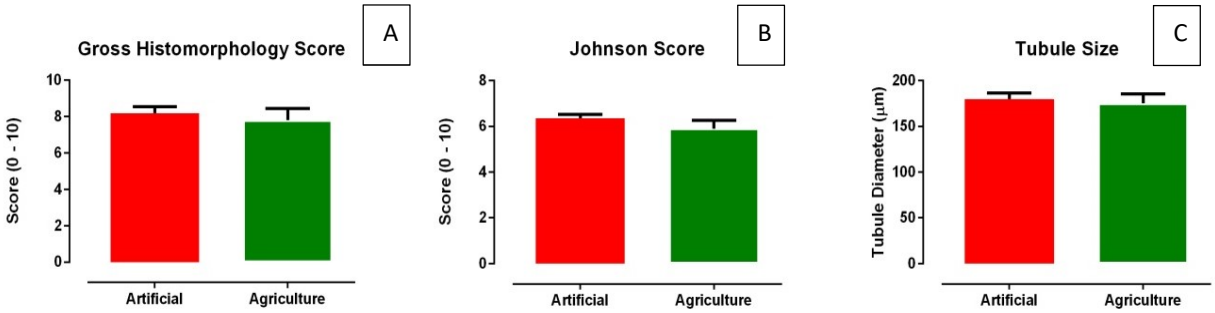


FIGURE 5-10 HISTOLOGICAL ANALYSIS OF UK TISSUE AND SCORING BY TWO CORINE LAND USE CATEGORIES: ARTIFICIAL AND AGRICULTURAL. NO STATISITICAL SIGNIFICANCE WAS SHOWN BETWEEN THE TWO CATEGORIES AND GROSS HISTOMORPHOLOGY SCORE, JOHNSEN SCORE AND TUBULE SIZE.

Analysis of Denmark testes tissue samples showed significant associations between histomorphometric scores and chemical concentrations which are shown in Table 5.5 and the observed relationships between the values is shown in Figure 5.11A - I. A significant positive correlation was found between gross histomorphology score and two congeners of PBDE (28, 153), Figure 5.11A-B, as well as four congeners of PCB (101, 118, 138, 153), Figure 5.11C - F.

TABLE 5-5 SIGNIFICANT ASSOCIATIONS SHOWN IN THE THREE HISTOLOGICAL SCORING METHODS OF GROSS HISTOMORPHOLOGY SCORE (GS), JOHNSEN SCORE (JS) AND TUBULE SIZE(TS) IN CANINE TESTES TISSUE COLLECTED IN DENMARK.

Assessment	Chemical	P value	R value	N value
GS	PBDE28	< 0.05	= 0.517	= 10
GS	PBDE153	<0.05	= 0.571	= 10
GS	PCB101	<0.05	= 0.507	= 10
GS	PCB118	<0.05	= 0.693	= 10
GS	PCB153	<0.05	= 0.586	= 10
GS	PCB180	< 0.05	= 0.536	= 10
JS	PCB52	<0.05	= 0.537	= 10
JS	PCB180	<0.05	= 0.476	= 10
TS	ΣPCB	<0.05	= -0.667	= 10
TS	PCB101	<0.05	= -0.659	= 10

The mean tubule size of the tissue collected from Denmark showed a significant negative correlation with ΣPCB ($P < 0.05$; $r = -0.667$; $n = 10$), Figure 5.13.C, as well as PCB101 ($P < 0.05$; $r = -0.659$; $n = 10$), Figure5.11 I. It is worth noting that these were the only negative correlations reported for tissue collected from Denmark. In the tissue collected from Denmark no significant correlation was found between DEHP concentrations and either gross histomorphology score, Johnsen score or tubule size.

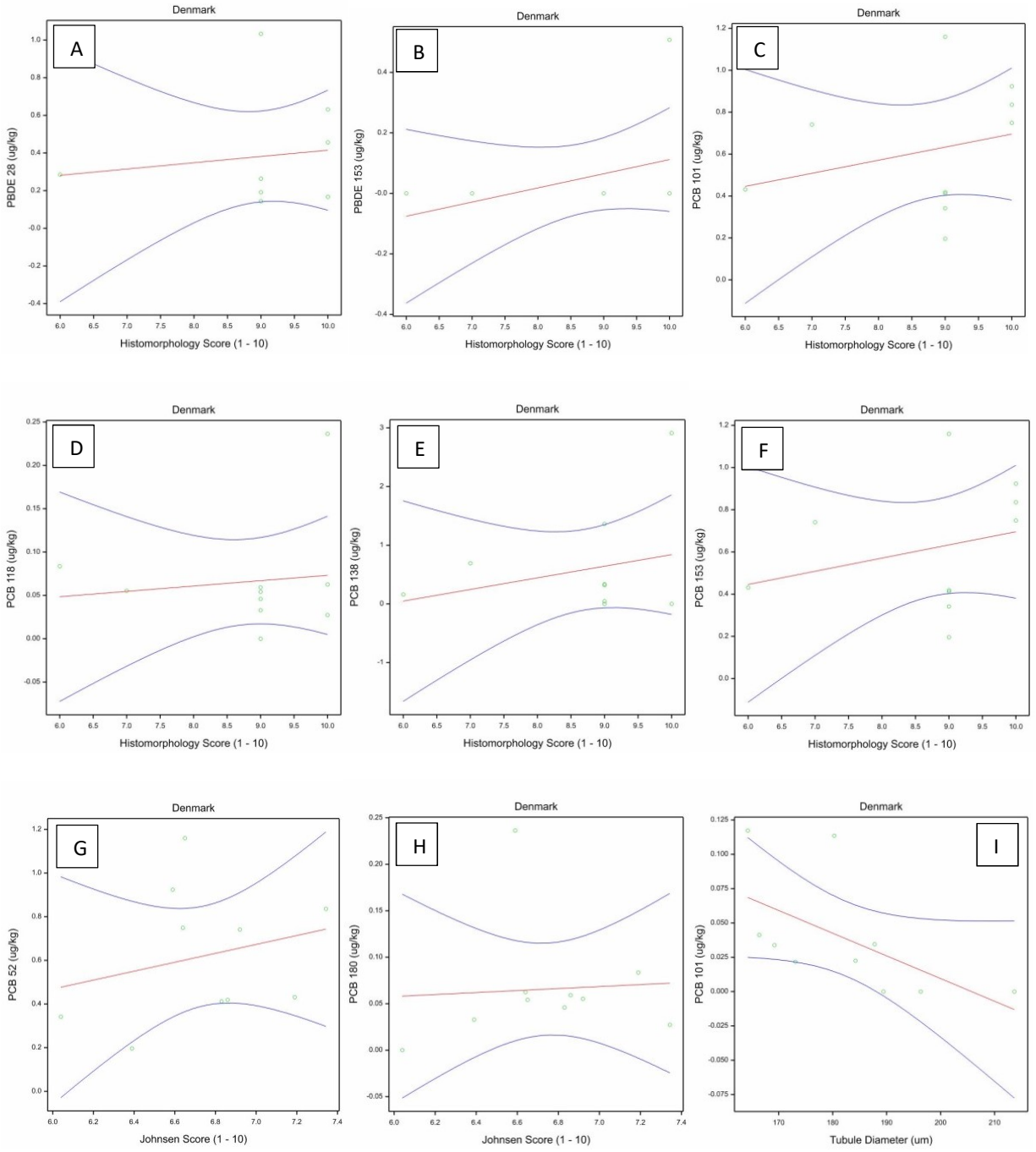


FIGURE 5-11 SIGNIFICANT CORRELATIONS (P < 0.05) BETWEEN PCB & PBDE CONCENTRATIONS AND HISTOMETRIC MEASURES OF TISSUE COLLECTED IN DENMARK. LINE REPRESENTS FITTED AND OBSERVED RELATIONSHIP WITH A 95% CONFIDENCE LIMITS SHOWN.

Samples from Finland showed a significant positive correlation between certain chemical concentrations and histomorphometric scores. Mean gross histomorphology score and two congeners of PBDE (PBDE28; PBDE100) were significantly and positively correlated. These relationships can be seen in Figure 5.12A and 5.12 F respectively. There were no significant correlations with Johnsen scoring and any of the chemicals. All significant correlation results

between histomorphometric scores and chemical concentrations in tissue collected in Finland are shown in Table 5.6.

TABLE 5-6 SIGNIFICANT ASSOCIATIONS SHOWN IN THE THREE HISTOLOGICAL SCORING METHODS OF GROSS HISTOMORPHOLOGY SCORE (GS), JOHNSEN SCORE (JS) AND TUBULE SIZE(TS) OF CANINE TESTES TISSUE COLLECTED IN FINLAND.

Assessment	Chemical	P value	r value	N value
GS	PBDE28	< 0.05	= 0.041	N = 10
GS	PBDE100	< 0.05	= -0.41	N = 10
GS	DEHP	< 0.05	= 0.498	N = 10
TS	ΣPBDE	< 0.05	= -0.539	N = 10
TS	PBDE28	< 0.05	= 0.522	N = 10
TS	PBDE47	< 0.05	= 0.539	N = 10
TS	ΣPCB	< 0.05	= 0.476	N = 10
TS	PCB138	< 0.05	= 0.472	N = 10
TS	DEHP	< 0.05	= 0.500	N = 10

Mean tubule diameter showed several significant correlations with the chemical concentrations. The ΣPBDE was negatively correlated with tubule diameter, shown in Figure 5.12E. Additionally, PBDE28, Figure 5.12B, reported a positive correlation and PBDE47, Figure 5.12C, reported a significant negative correlation with mean tubule size.

There were significant associations between tubule size and chemical concentrations of PCBs. Both ΣPCB ($P < 0.05$; $r = 0.467$; $n = 10$), Figure 5.13C, and PCB138 ($P < 0.05$; $r = 0.472$; $n = 10$) showed a significant positive correlation with mean tubule size as shown in Figure 5.12F.

DEHP showed a significant association with both mean gross histomorphology score ($P < 0.05$) and Mean tubule size ($P < 0.05$), Figure 5.14C - D, but no significant correlation was found between DEHP and Johnsen score ($P > 0.05$).

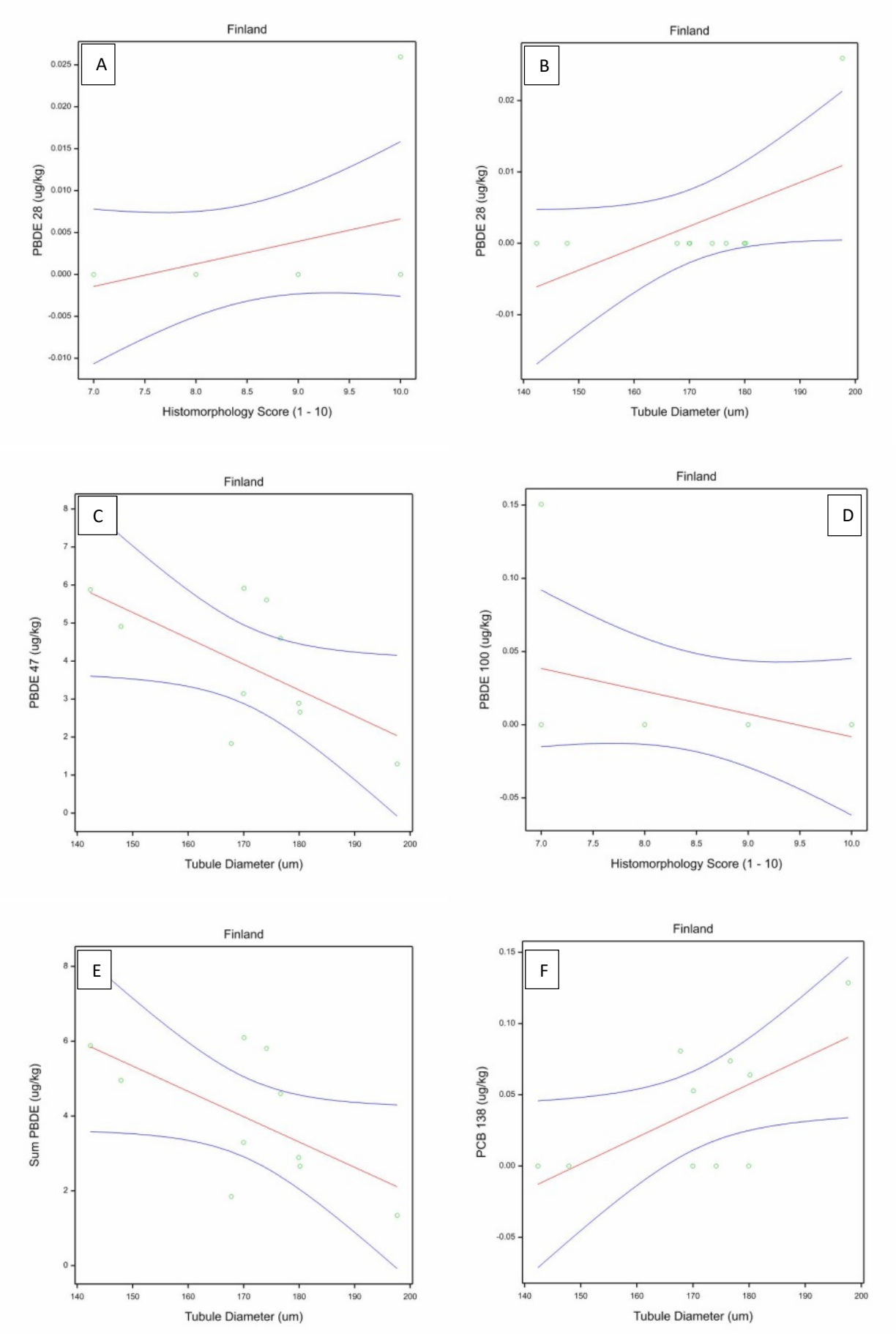


FIGURE 5-12 SIGNIFICANT CORRELATIONS (P < 0.05) BETWEEN PCB & PBDE CONCENTRATIONS AND HISTOMETRIC MEASURES OF TISSUE COLLECTED IN FINLAND. LINE REPRESENTS FITTED AND OBSERVED RELATIONSHIP WITH A 95% CONFIDENCE LIMITS SHOWN.

It is worth noting that tubule diameter measures for all countries correlated significantly with ΣPCB. Figure 5.13A - C shows the slope of the relative linear regression lines across the three countries for this measure. Tissues from the UK and Finland showed a positive correlation (r = 0.239, r = 0.467 respectively), Figure 5.13A & B. Tissue from Denmark, Figure 5.13C, showed a negative correlation between tubule diameter and ΣPCB (r = -0.667).

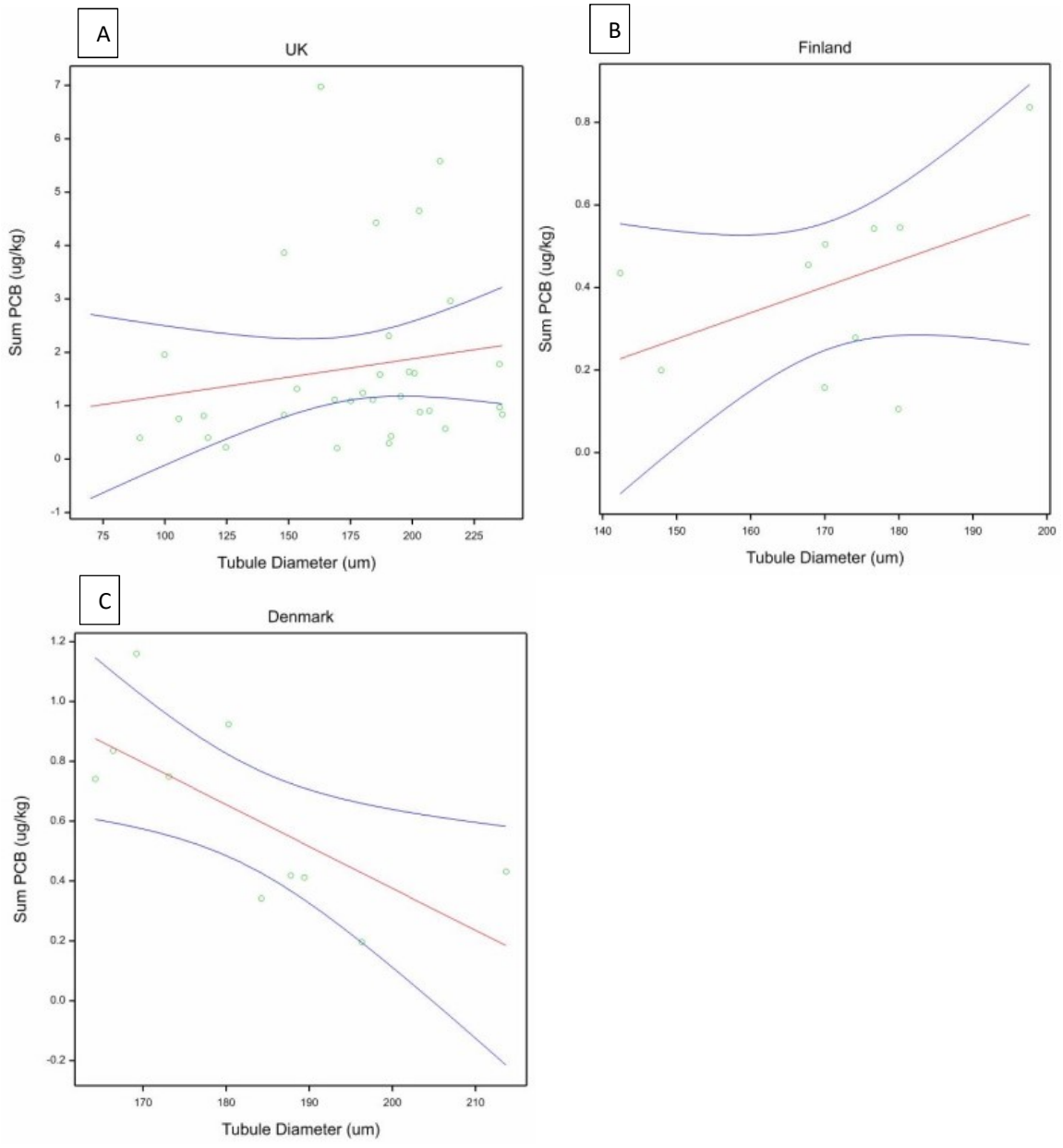


FIGURE 5-13 SIGNIFICANT CORRELATIONS (P < 0.05) BETWEEN ΣPCB TUBULAR DIAMETER FOR ALL THREE COUNTRIES: A) UK, B) FINLAND, C) DENMARK. LINE REPRESENTS FITTED AND OBSERVED RELATIONSHIP WITH A 95% CONFIDENCE LIMITS SHOWN.

The results of associative analysis for DEHP and histomorphometric scores identified significant correlations for tissue from Finland and the UK only. There were no significant correlations between DEHP and any of the histomorphometric scores for tissue collected from Denmark. The relationship, for tissue collected in Finland, between DEHP and gross histomorphology and tubule diameter was a positive correlation as shown in Figure 5.14C - D. Figure 5.14 A - B demonstrates the positive relationship reported between DEHP and gross histomorphology and tubule diameter for tissue collected in the UK.

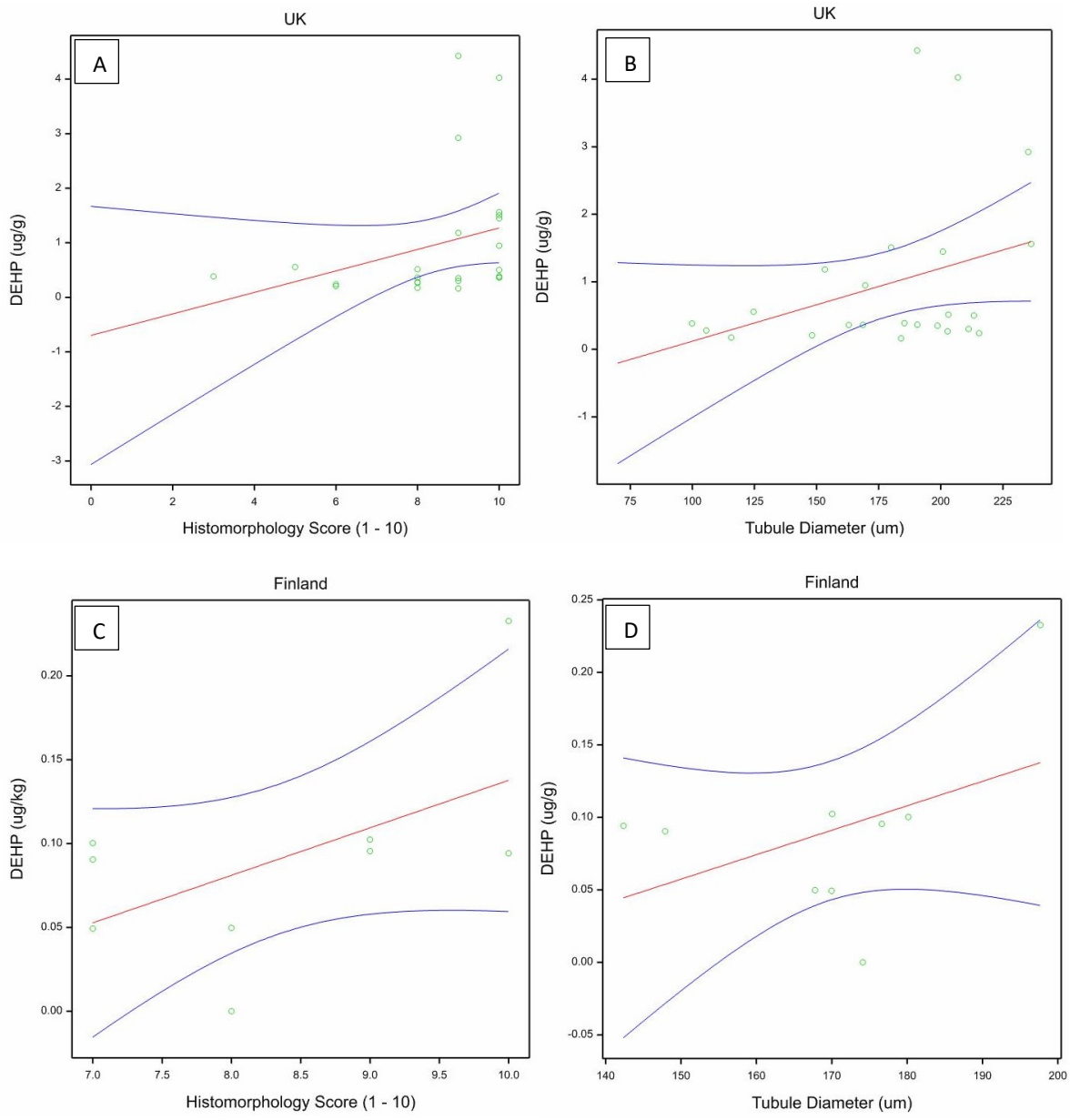


FIGURE 5-14 SIGNIFICANT CORRELATIONS (P < 0.05) BETWEEN DEHP AND BOTH HISTOMOROLOGY SCORE AND TUBULAR DIAMETER FOR: A-B) UK, C-D) FINLAND. LINE REPRESENTS FITTED AND OBSERVED RELATIONSHIP WITH A 95% CONFIDENCE LIMITS SHOWN.

The canine testes tissue collected from the three countries of UK, Denmark and Finland showed no significant differences in the gross histological score, Johnsen score or tubule size when statistically analysed for variance across the three sample cohorts. The mean score or measure for each country (±SEM) are shown in Figure 5.15.

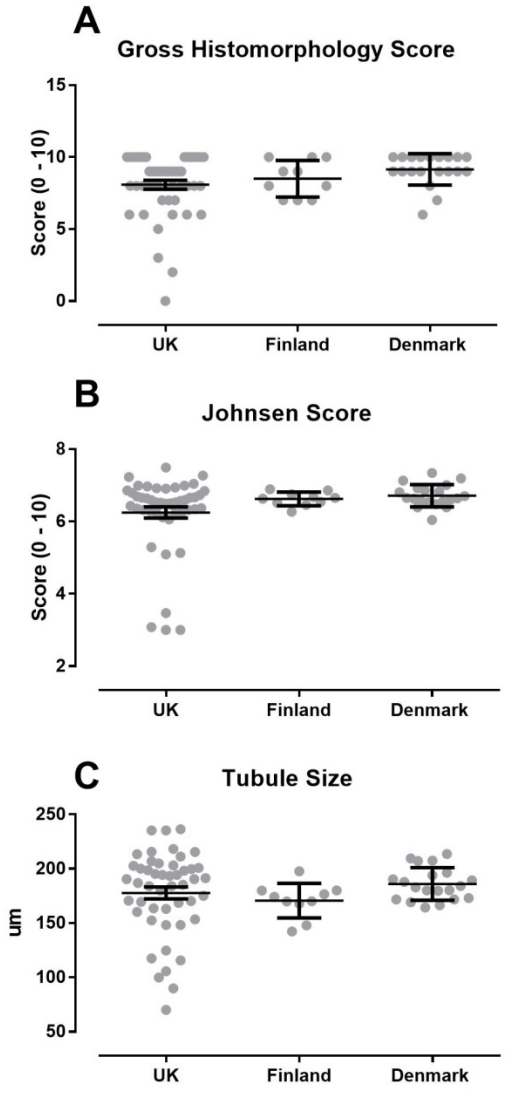


FIGURE 5-15 HISTOLOGICAL ASSESSMENT OF TISSUE SAMPLES COLLECTED ACROSS THREE COUNTRIES: UK, FINLAND, DENMARK. A) MEAN GROSS HISTOMORPHOLOGICAL SCORING OF THE TESTES TISSUE ACROSS THE THREE COUNTRIES SHOWING NO SIGNIFICANT DIFFERENCE. B) MEAN JOHNSEN SCORING OF THE TESTES TISSUE SHOWING NO SIGNIFICANT DIFFERENCE. C) MEAN TUBULE SIZE OF THE TESTES TISSUE SAMPLES COLLECTED SHOWING NO SIGNIFICANT DIFFERENCE ACROSS THE THREE COUNTRIES.

5.3.1 IMMUNOHISTOCHEMICAL ANALYSIS

There was no significant association identified between the percent of area stained for CYP11a1 and concentrations of seven PCB congeners, seven PBDE congeners or DEHP in the tissue collected from the UK. Although there was a significant negative correlation reported between the per cent area stained for CYP11a1 and Johnson score ($P < 0.05$; $r = -0.543$; $n = 32$) as well as tubule size ($P < 0.05$; $r = -0.411$; $n = 32$).

The samples from the UK showed no significant differences in the area stained for CYP11a1 between three geographic regions of England (Figure 5.16A). Additionally, there was no significant differences in the area stained when the tissue were grouped by two CORINE land use categories (Artificial and Agricultural) as shown in Figure 5.15B.

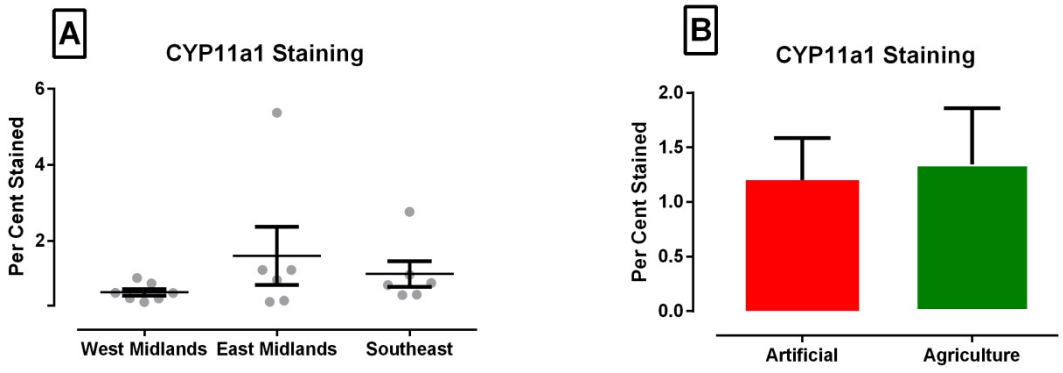


FIGURE 5-16 AREA STAINED FOR CYP11A1 IN TISSUE SAMPLES COLLECTED IN THE UK. A) TISSUE SAMPLES GROUPED BY GEOGRAPHIC REGIONS IN THE UK. NO SIGNIFICANT DIFFERENCE WAS IDENTIFIED ($P > 0.05$). B) TISSUE SAMPLES FROM THE UK GROUPED BY CORINE LAND USE CATEGORIES. NO SIGNIFICANT DIFFERENCE IDENTIFIED ($P > 0.05$). (BAR: ±SEM).

Tissue collected from Denmark and stained for CYP11A1 showed a significant correlation between area stained and concentrations of ΣPBDE and the individual congener PBDE47 (<0.05; t = -0.810; n = 10: $P < 0.05$; t = -0.714; n = 10 respectively). The observed relationship between ΣPBDE and PBDE47 is shown in Figure 5.17A & B. There was one other chemical which had a significant correlation with CYP11a1 area stained (PCB52: $P < 0.05$; t = 0.520; n = 10).

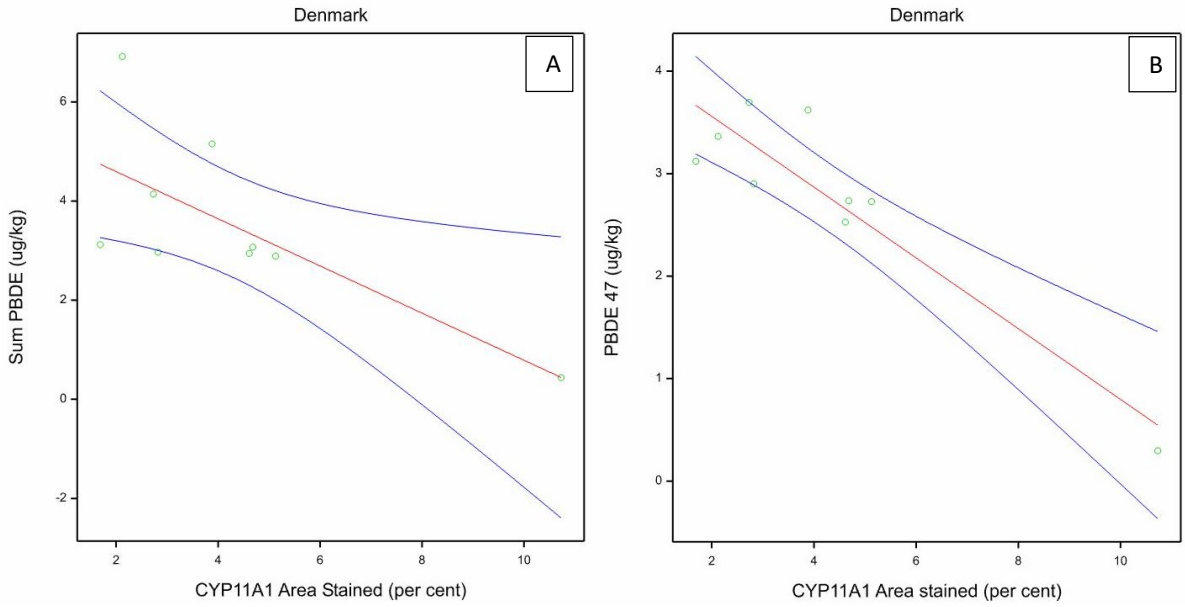


FIGURE 5-17 SIGNIFICANT CORRELATIONS (P < 0.05) BETWEEN DEHP AND CYP11A1 AREA STAINED IN TISSUE COLLECTED FROM DENMARK. A) RED LINE REPRESENTS FITTED AND OBSERVED RELATIONSHIP BETWEEN ΣPBDE AND AREA STAINED FOR CYP11A1. B) RED LINE REPRESENTS FITTED AND OBSERVED RELATIONSHIP BETWEEN PBDE47 AND AREA STAINED FOR CYP11A1. BLUE CURVED LINE REPRESENTS A 95% CONFIDENCE LIMITS .

Tissue collected from Denmark and stained for CYP11A1 reported no significant correlation with any of the three other histological measures: gross histomorphology, Johnsen score, tubule diameter.

When tissue samples collected from Finland were analysed statistically for an association between per cent area stained for CYP11A1 and chemical concentrations of seven congeners of PCB, PBDE and DEHP, no significant associations were found. A single histological assessment (tubule size) correlated significantly with the area stained for CYP11A1 ($P < 0.05$; $r = 0.515$; $n = 10$).

The area stained for CYP11A1 was significantly different across the three national regions ($P<0.0001$). Figure 5.17 shows the mean area stained (±SEM) for the three countries.

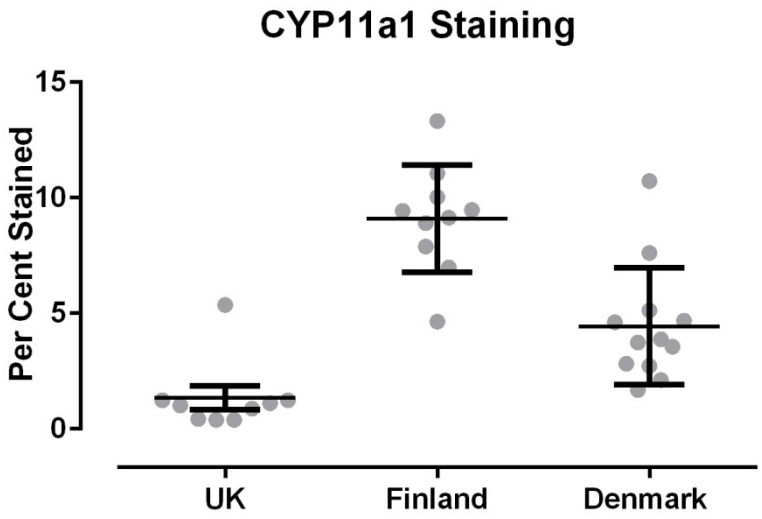


FIGURE 5-18 PER CENT AREA STAINED FOR CYP11A1 IN TISSUE COLLECTED FROM THREE NATIONAL REGIONS. A SIGNIFICANT DIFFERENCE ($P < 0.0001$) WAS FOUND ACROSS THE THREE COUNTRIES. SAMPLE SIZE N = 9, 10, 12 RESPECTIVELY, BAR ±SEM.

5.4 DISCUSSION

A range of significant correlations, indicating both positive and negative relationships, were found between chemical concentrations and the three histological measures: gross histomorphology, Johnsen score and tubule size. These correlations do not demonstrate causation but provide points for discussion. The concentrations of the chemical group PCB showed significant correlation with histological measures at a higher frequency than the other two chemical groups. This result further proves that PCB is worth further investigation especially mechanistic research using this exemplar pollutant. More specifically across the three countries results demonstrated, congeners of PCBs reporting significant correlations six times against histomorphometric scores in the UK (Gross Histomorphology: Congener 101, 118, 138, 153; Tubule Diameter: Congener 101, ΣPCB), eight times in tissue from Denmark (Gross Histomorphology: Congener 101, 118, 153, 180; Tubule Diameter: Congener 101, 180, ΣPCB; Johnsen Score: Congener 52) and two times in Finland (Tubule Diameter: Congener 138, ΣPCB). The number of significant correlation for PBDE and DEHP across all four countries was 10 and 4 respectively. If any generalisations can be taken from this result, it may suggest that PCB, even though it has been banned since 1977 (Rasmussen, Schrank, & Williams, 2014), is an environmental pollutants which may perturb development and function of the mammalian testis. The results suggest the same can be said for PBDE and DEHP although to perhaps a lesser extent.

If it is accepted that PCBs are having a detrimental effect on testes development and function, negative correlations between PCB concentrations and histomorphometric scores would be expected. This was only the case for ΣPCB, PCB101 and tubule diameter in tissue collected from Denmark only. All other significant correlations between the concentrations of PCB congeners and histomorphometric scores in the three countries indicate a positive

relationship. A straightforward mechanistic action, following a linear dose response does appear to not explain the results for these correlative analyses. This result demonstrates the need for complementary mechanistic studies into the effect of these environmental pollutants.

When using quantitative morphometric analysis, such as Johnsen scoring, as an indicator of testes function and spermatogenesis (Peters et al., 2000) a negative correlation with chemical concentrations of substances like PCBs would be expected. Indeed, PCBs are reported to have toxic reproductive effects on the testes (Golub et al., 1991), would be expected. In contradiction to this expectation, Johnsen scores showed a significant positive correlation with chemical concentrations of ΣPCB and congener PCB 101 for tissue collected in the UK. The tissue from the UK reporting this result was also had the highest mean PCB concentration of the three regions. This result must be considered in light of the non-linear dose response hypothesis and therefore the effects may not be dose dependent in a classic toxicological way.

Seminiferous tubule diameter is associated with testes function and spermatogenesis (Wing & Christensen, 1982, Ogbuewu eta al., 1989, Regadera et al., 2001, loeje, 2009) and is a credible measure of testes function (Sah et al., 2015). Consequently, this parameter warrants further investigation as tool for assessing developmental differences across groups and regions.. As stated previously, the expectation was that tissue exposed to reproductive toxins and endocrine disruptors would exhibit negative correlations with histomorphmetric assessment. With respect to tissue from Denmark, this was the case with the result for ΣPCB and PCB47. Contradictorily, significant correlations from Finland and the UK suggested a positive relationship. Significant correlations between ΣPCB and tubule size were found across tissue collected from all three national regions. Interestingly only Denmark showed a significant negative correlation but this was a very strong negative correlation ($r = -0.829$). Overall tubule

size seemed to be negatively correlated with PBDE in tissue collected in Finland only. This result is not clear as ΣPBDE and PBDE47 were negatively correlated in spite of a positive correlation reported for PBDE28 and tubule diameter. If a conclusion for this result was to be made then ΣPBDE and PBDE47 were more strongly correlated with PBDE concentrations and more weight would therefore be given to these negative correlations. When measuring tubule diameter it should be noted that optimal diameter is unknown and examining this as a linear relationship may be a blunt tool. Also, tubule diameter changes during the stages of spermatogenesis which may affect these results. These two factors need further investigation to fully understand their effect on this type of metric but the results reported herein are indicative of a potential perturbing effect of PBDE on testes development as reflected by histomorphometric measures.

The discrepancy in the relationships reported between chemical concentrations and histomorphometic scores across national geographic regions demonstrate the subtle and the complex effects of these chemicals in a real world environment. The dogs, from which tissue was collected, were exposed through foetal development and adult life to a mixture, or cocktail, of environmental pollutants. To attempt to elucidate a mechanistic effect is not the focus of this aspect of our research. What are interesting from the data are the geographic differences in the results from tissues collected in regions with a historically relevant track record of reproductive health and fertility.

It is worth noting that Denmark is reported to have lower reproductive health and sperm quality than other European countries (Jorgensen, 2001). The correlations when viewed by country and in consideration of historically relevant sperm quality data (and other aspects of TDS) provide a more interesting and broader picture. Tissue collected from Denmark, although not having the highest mean concentration of PCB, reported the most significant

number of correlations between these chemicals and histomorphometric scores (n = 8). This is also the country with the lowest reported sperm quality (Jorgensen, 2001; Punab et al., 2002). Additionally, Denmark was the only country to report a negative correlation between congeners of PCB and tubule size. Tissue from Denmark also differed from the other two countries in that tissue collected in both the UK and Finland showed a positive correlation between gross histomorphology scores and the same four congeners of PCBs (101, 118, 138, 153). Tissue from Denmark showed no significant correlations between these four congeners and gross histomorphology. So the broader interpretation of the data, especially for PCBs, would indicate that tissue collected from Denmark is responding differently to tissue collected from other countries and that this may reflect exposures to different pollutants.

Measures from the tissue collected in Finland only reported a correlation between one histological measure, tubule diameter, and concentrations of PCBs (congenersPCB138, ΣPCB). This is interesting, as tissue from Finland, as reported previously (Chapter 4.3), had the lowest mean concentration of ΣPCB and the highest mean concentrations ΣPBDE. This result suggests that lower concentrations of PCB have fewer significant correlations between histological scores in accordance with the concentrations of this chemical group in this region. Additionally, Finland showed a correlation between the chemical group PBDE and gross histomorphology (Congener 28, 100) and tubule diameter (Congener 28, 47, ΣPBDE). These five significant correlations compare to two and three in the UK and Denmark respectively. This again reflects the mean concentrations of PBDE found in tissue collected in these countries with Finland having the highest and UK having the lowest. Finland reported better semen quality and lower rates of reproductive developmental disorders (Jorgensen, 2001) when compared to other countries. This may suggest threshold concentrations of PBDEs,

within the range found in the environment, having an effect on fertility in proportion to the concentrations found in tissue.

Concentration of DEHP correlated with two measures of histological assessment in two countries: UK and Finland. Intriguingly these two measures (gross histomorphology, tubule size) were the same in both countries. This significant correlation between DEHP concentrations and gross histomorphology and tubule diameter was found in both the UK and Finland may indicate this chemical acts in some mechanistic way on testes development and the similarities found across two regions lend credibility to this conclusion. The highest mean concentrations of DEHP was reported in tissue collected in the UK (0.96537µg/g) and Finland (0.0904 µg/g) the lowest. Denmark (0.2370µg/g) tissue had a concentration, higher than, but similar to that found in Denmark. This constitutes another reminder of Denmark seemingly acting differently in response to individual chemicals as a single chemical acting in a dose response would expect the similarity in effect at similar concentrations: i.e. Denmark and Finland. The similarity in histomorphometric scores for the chemical DEHP in our results is found in tissue at either end of the concentration range.

The apparent conundrum between concentrations of DEHP and histomorphometric scores is an example of the complexities of the effects of endocrine disruptors and a reminder of the NMDR and low dose hypothesis of the effects of these substances (Vandenberg et al., 2012). This point is reinforced by the work of researchers reporting that the highest dose of DEHP (500 000µg/kg/d) was not significantly different from the control group (0 µg/kg/d) (Do, Stahlhut, Ponzi, Vom Saal, & Taylor, 2012). The exact physiological effect of DEHP and other chemicals of this group (phthalate acid esters) are not exactly known but many researchers have suggested an oestrogenic or antiandrogenic mechanism (Fisher, 2004; Pant et al., 2011; Pocar et al., 2012). More specifically the action of DEHP has been reported to alter the

interaction of foetal gonocytes and Sertoli cells (Fisher, 2004) as well as a reduction in Leydig cell numbers. This type of effect would subsequently have an impact on male reproductive development if exposure occurred *in utero*, as has been demonstrated in rats exposed during the sensitive 'male programming window' to phthalates (Scott et al., 2008) but could also act via the ER or the AR.

As a measure of Leydig cell area in the testes, the first steroidogenic enzyme involved in this process is considered to be a strong indicator for this measure (Gilibili et al., 2014). Therefor CYP11a1 was used as an indicator of testes Leydig area and these results suggest variation across national geographic areas as a significant difference in area stained was found between tissue collected from UK, Finland and Denmark. If this is the case, the causation for this difference in Leydig cell area is again strongly suggestive of an environmental aetiology as lifestyle is not a factor in canine physiology as could be suggested of humans. This reinforces the hypothesis that the geographic variation in components of TDS, and other measures of reproductive health are effected by environment more than lifestyle. Additionally, tissue collected from Denmark reported a negative correlation with CYP11A1 testicular area stained and concentrations of ΣPBDE and PBDE47. This is another example of a difference between tissue from Denmark and the other two countries.

Conclusion

Conclusions based on correlative associations need to be made with a consideration of the limitations of this type of analysis. A careful examination of the data presented in Figures 5-8, 5-11, 5-12 all show how one or two outlying values can create a statistically significant correlation. These 'rogue' values may create an artefact which is suggestive of a relationship and therefore should be viewed with caution. The results showing differences histomorphometric measures across the three geographic regions are perhaps more important in elucidating the effects of environmental chemicals.

In consideration of the comments above, the seemingly discrepant results showing positive relationships between chemical concentrations are difficult to explain if a normal dose response is expected but with EDs and other environmental chemicals a non monotonic dose response is not untypical (Vandenberg et al., 2012). The results for ΣPCB is an example of this phenomenon as this chemical correlated positively with tubule size when tissue from Finland and the UK were analysed but a negative correlation for these factors was reported from tissue from Denmark. The concentrations of ΣPCB reported in testis tissue across the three countries showed tissue from the UK having the highest mean concentrations followed by Denmark and Finland. This suggests a possible effect of PCBs but is more strongly suggestive of additional factors contributing to these effects,

Morphometric measures of histological structures of the testes may be an indicator of development and function. These measures also allow for quantitative and semi quantitative analysis of the testes and associations between these metrics and others may indicate a relationship but perhaps not direct causation. The weight of evidence of the histomorphological assessment of the testes tissue, as well as the national differences reported, suggests an association between environmental chemical burden and measurable

aspect of testes structure and function. The exact associations and underlying mechanism requires further elucidation.

One of the strongest pieces of evidence supporting and environmental aetiology for the reported negative temporal trends in male sperm quality, as with other aspects of human TDS, is the variation of results across geographic regions and between countries. The overall results of the histomorphometric and immunhistochemical analysis of the testes tissue suggests that there are differences across the three countries (specifically, the regions with in these countries) from which tissue was collected and analysed. Denmark, the country with historically significant record of relatively poor fertility, differed from other two countries in a number of results. This further supports the hypothesis of an environmental aetiology for trends seen in male fertility. Our results, which do not clearly suggest a straightforward mechanistic explanation for this phenomenon, do suggest that exposures to chemicals in Denmark (the full mixture /combined effect model) may create a 'perfect storm' resulting in the reported lower fertility in this country.

This suggests that environmental factors, such as pollutant concentrations which differ across these regions, effect reproductive development, testes structures and ultimately fertility is supported by these results. It has been hypothesised that the reported negative temporal trends and geographic variation in reproductive development and fertility in the human have been linked to lifestyle factors (Russ Hauser, Chen, Pothier, Ryan, & Altshul, 2003; Jorgensen, 2001; Jurewicz et al., 2013; Sharpe & Skakkebaek, 2008). As a contributing factor lifestyle is less relevant when considering canine samples, so results with this factor removed, as is the case with the canine model, are suggestive of causative factors outside of lifestyle influences. Many other researchers suggest that lifestyle alone cannot be fully responsible for the negative temporal trends and geographic variation reported in male sperm quality (Le Moal et

al., 2014b; Sharpe & Skakkebaek, 2008; Tsarev et al., 2005) Therefore overall this suggest that environment, outside of lifestyle factors, is more likely to be responsible for geographic variation in reproductive health and fertility. The canine model is a stronger indicator that environmental factors as contributing to the negative trends seen in male fertility and development.

The mechanistic effects of PCBs, PBDEs, DEHP cannot be elucidated by the results of our research to this point. The action of these chemicals may be via the endocrine system, as with EDs, of some other mechanism. Insight into these mechanisms may be achieved via exposures of testes tissue and spermatozoa to environmental chemicals. The results reported in this chapter, although suggestive of a possible relationship, cannot credibly be linked to mechanistic actions. Therefore the next chapter will examine, by culturing testis tissue and spermatozoa with exemplar chemical pollutants, the direct mechanistic effect of exposures on fertility and reproductive function.

Chapter 6 TISSUE AND SPERM CULTURES

6.1 Background and Literature

One of the challenges when attempting to draw conclusions about the concentrations of EDs or other chemicals in tissue or serum is the variability of these concentrations. In individual samples, serum PCB concentrations are reported from a low of below limits of detection to a high of 240 ng/g in human serum (Anna Rignell-Hydbom et al., 2007). The mean concentration found in testes tissue from the UK reported in chapter 3.3.3. was 0.3251 µg/kg. Data presented in chapter 4.3 illustrates that chemical pollutants have been found in varying level in canine testes tissue and that there are differences across intra national and international geographic regions in the UK, Finland and Denmark. Other researchers have also found differences in concentrations of chemical pollutants across intra national geographic boundaries in non-human species, e.g. sheep (Rhind et al., 2011). Although variable in concentrations, environmental chemical pollutants, specifically PCBs which are known to be ubiquitous in all environments globally (Mochungong & Zhu, 2015), have been found in testes tissue and other canine media, as reported here. Also, phthalate esters in the form of DEHP have also been found in these same samples tested. These chemical substances are recognised as reproductive toxins and also recognised as endocrine disruptors (ED) (WHO, 2000). One hypothesis is that human TDS has an environmental aetiology through exposures to EDs (Katrine Bay et al., 2006) and strong evidence of a canine TDS has been demonstrated by our research through reduced semen quality and increased cryptorchidism (chapter 3.3.1) and suggested by others (Grieco et al., 2008).

The reported negative temporal trends in human semen quality and reproductive health have been suggested as having an environmental aetiology (Katrine Bay et al., 2006; Kalfa et al., 2015, 2009; Saalu & Osinubi, 2009; Skakkebaek et al., 2001). There is also support from wildlife models suggesting environmental chemicals effect reproductive development

(Edwards, Moore, & Guillette, 2006; Facemire et al., 1995; Guillette et al., 1985; Orton & Tyler, 2014) . Based on evidence from other species, it could be hypothesised that canine testes development, function and steroidogenesis may be perturbed *in vitro* by exposure to known EDs at environmentally relevant concentrations. *In vitro* culture of testes tissue and spermatozoa with low dose, environmentally relevant concentrations of known EDs may demonstrate an association between chemical pollutant concentrations and reproductive function.

6.1.1 LOW DOSE AND NON MONOTONIC DOSE RESPONSE CURVE

Traditional toxicological investigations are often based on the assumption that 'the dose makes the poison'. This convention may not fully explain the mechanism of action of environmental pollutants as the endocrine effect of these chemicals can have a non-linear dose response curve (WHO, 2013). Many of the chemicals identified as endocrine disruptors also are effective at low doses (Borges et al., 2012) compared to, for example, effects found at toxic concentrations such as those used in conventional LD_{50} testing. This emphasises the importance of environmental concentrations, as these compounds may be most active at relatively low concentrations and perhaps with some only active at low concentrations. Although a minority of researchers reject a low dose effect of EDs on humans (Nohynek, Borgert, Dietrich, & Rozman, 2013) there are range of studies which support this hypothesis. The low dose effect theory would negate the commonly used Lowest Observable Effect Concentrations (LOAEL) and Non Observed Adverse Effect Concentrations (NOAELS) criteria often used when risk assessing exposure limits. Since these are relatively higher doses when compared to environmental concentrations, these values for safe concentrations have been criticised as outdated. In consideration of this it is interesting to note that DEHP and PCB 153

are both reported to have background concentrations in humans below the NOAEL/LOAEL limits of DEHP: 5.8mg/kg (Vandenberg et al., 2012); PCB: 5µg/g (Giesy & Kannan, 2008).

The importance of using concentrations which relate to body concentrations for research is currently considered a credible methodology and will be more demonstrative of biological/environmental effects rather than toxicological effect. Real life exposures are chronic and at low environmental concentrations, therefore methodologies designed to investigate true environmental effects should attempt to replicate these exposures. Many reports now are using methodologies supported by specific background concentrations of particular chemicals at relatively low environmental exposure concentrations (Melnick, 1999) which is a progression from traditional toxicological research.

The chemical pollutant PCB, both mixtures (e.g. Aroclor 1221) and single congeners, have been demonstrated by a number of researchers to have an effect when animals and humans are exposed at low doses *in vivo* and therefore reflective of environmental exposures (Brouwer et al., 1999; Buckman, Fisk, Parrott, Solomon, & Brown, 2007; Dickerson, Cunningham, Patisaul, Woller, & Gore, 2011; Salama et al., 2003; Tanaka et al., 2011). In addition, researchers reporting the effects of exposure to the phthalate DEHP have utilised methodologies in recognition of both Non Monotonic Dose Response Curve and low dose effects (Do et al., 2012; Hsu et al., 2014; Hirai et al., 2015)

6.1.2 Specific effects demonstrated on testes tissue and spermatozoa

Due to its persistence, lipophilia and slow biodegradation, a background burden of PCB is carried by all animals on the biosphere including humans (Schettgen et al., 2015). These body concentrations are considered environmental exposure concentrations. Due to its ubiquity, ban as a carcinogen and identification as one of the compounds which cause harm to the

function of the reproductive tract, PCB is a high priority pollutant. Marine mammals are an interesting case study as they have seen to have dramatic developmental disorders of the reproductive tract. St Lawrence Beluga whales have been seen presenting true hermaphroditism (Deguise et al., 1994) and precarcinogic tumours (De Guise et al., 1995). Both of these conditions have been partially contributed to exposures to organochlorines and other pollutants. Not surprisingly, Killer Whales (*Orcinus orca*) as the top predator in the marine trophic cascade contains concentrations of organochlorines and PBCs which are reported to be in excess of all safe toxicity concentrations (Jepson et al., 2016) .

The congener PCB 153, a proxy for total ΣPCB (Verner et al., 2015), has been identified as an endocrine disruptor (Bonefeld-Jørgensen et al., 2001; Chu et al., 1996; Ness et al., 1993; Patnode & Curtis, 1994; Schantz et al., 1997) and has been shown to have an association with decreased sperm motility across a range of species (Bush, Bennett, & Snow, 1986; Dallinga, 2002; Russ Hauser et al., 2003; Meeker et al., 2011; Jonas Richthoff et al., 2003). The exact mechanisms of endocrine disruption and sperm motility are most likely two separate, albeit both directly linked to fertility, factors in the overall reproductive toxicity of this compound.

There is a paucity of research, using *ex vivo* exposure models, examining the effects on sperm cultured with specific congeners of PCB. One study indicated that the concentrations of three PCB congeners (2,4,5,2'4'5' hexachlorobiphenyl (PCB 153) 2,4,5,2'3'4' hexachlorobiphenyl (PCB 138) and 2,4,5,3'4'-pentachlorobiphenyl (PCB 118)) are inversely correlated with human sperm motility (Bush, Bennett, & Snow, 1986). It is also worth noting that congener 118 was found in testis tissue and food in our results. Hauser (2002) also suggested that PCBs have a negative relationship to semen quality, specifically motility. This study was pilot work with a low sample size but is supported by the work of Bush (1986). Additional work by Hauser (2003) showed an inverse dose response relationship with certain PCBs and human sperm motility.

Another negative effect of PCB reflects is association with a decrease in sperm chromatin integrity. Some workers have had inconclusive results (Rignell-Hydbom et al., 2005; Rignell-Hydbom et al., 2007; Spanò et al., 2005) but others report a slight decrease in sperm chromatin integrity in adult humans but also suggest that other factors may be involved (Spano et al 2005).

6.1.3 DEHP AND REPRODUCTIVE FUNCTION

A negative correlation between phthalate exposure and sperm function (Niraj Pant et al., 2008) has been reported in humans. This research has direct implications for this species but the majority (73% as determined by literature review (Albert & Jégou, 2014)) of our knowledge of phthalate exposure and testes function comes from a rat *in vivo* model. This laboratory based rodent research is useful as a model but as we are not concerned with temporal trends in rodent fertility, a step away from species of interest. Additionally the majority of this rodent research is based on methodologies using relatively high, non environmentally relevant doses (Albert & Jégou, 2014). In terms of mechanism to explain the effect of DEHP on reproductive function, most of the research suggests an anti androgenic mechanism (Bornehag et al., 2015; Gray et al., 2006; Jones et al., 2014) for the dysgenesis and dysfunction of the testes when exposed *in utero* to phthalates. These explanations are limited when it comes to determining hormonal perturbations as systemic artefacts cannot be ruled out. To isolate the effect to specific target tissue culture systems using micro dissected testes explants can be more directed in terms of concentrations and duration of exposure to the target organ.

6.1.4 DNA Fragmentation as a measure of fertility

DNA fragmentation is becoming a more recognised indicator of potential fertilization (Smith et al., 2013) and therefore ultimate fertility (Brahem et al., 2012; Sonia et al., 2012; Moazzam, Sharma, & Agarwal, 2014) The supposition that DNA integrity is linked to successful fertilisation (Evenson & Wixon, 2006) has been established by a number of researchers but has yet to become a routine measure of fertility like total sperm output, motility and morphology (Fernández et al., 2005). There are several methodologies put forward to measure DNA fragmentation as indicative of fertility. These include Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (Tunel) (Guneli et al., 2008; Tsounapi et al., 2016), Comet assay (Berlinguer et al., 2009; Mathieu-Denoncourt, Wallace, de Solla, & Langlois, 2014; Toft et al., 2014) and Sperm chromatin structural assay (SCSA) (Fernindez et al., 2003).

The ranges of assays all have in common the quantification of breaks in DNA as an indicator of cellular viability. More directly, it is a measure of necrozoospermia and cellular apoptosis as both of these are associated with DNA fragmentation (Zhang & Xu, 2000). The importance of DNA fragmentation for research into environmental pollutants and sperm quality has been demonstrated by reported associations reported between air pollution (Rubes et al., 2005), cigarette smoking (Sepaniak et al., 2006), other environmental toxicants (Evenson & Wixon, 2005) and sperm DNA fragmentation.

The studies described in this chapter have been designed to investigate the effects of environmental chemicals found in adult dog testis on testicular endocrine function and on sperm viability and motility. This section of work was designed to further consolidate the original working hypothesis which stated that declining sperm quality in dogs (reported in chapter 3) may reflect acute and/or chronic exposure effects of exposure to environmental chemicals. Specifically, the experimental objectives of chapter 6 are as follows:

1. Investigate short term (acute) effects of testicular concentrations of PCB153 and DEHP on spermatozoa culture *in vitro*. End point measurements were motility and sperm DNA fragmentation.
2. Determine the effects of PC153 and DEHP on testicular hormone secretion using a novel canine testis explant culture method.
    a. This objective required the design and validation of a canine testis explant culture system and the demonstration of testosterone secretion in response to the addition of luteinizing hormone.

6.2 METHODS

A canine testis explant culture system was optimized as described in Chapter 2.10.1 and this optimized protocol was used for the culturing of Canine testes (n = 8), residual tissue from routine castrations were collected from a veterinary hospital (Pride Veterinary Centre, Derby) associated with the University of Nottingham School of Veterinary Medicine and Science. Tissue was transported in a thermal flask in pre warmed (37°C) transport medium (DMEM, 10% bovine foetal serum) within 1 hour of collection back to the University laboratories. Full methodologies for tissue collection, preparation and culture are given in chapter 2.2.

The tissue was cultured for a 24 hour incubation period based on optimization results and other published work (Fowler et al., 2007). Tissue was exposed to two concentrations of PBC 153 and DEHP and three concentrations of Luteinizing hormone (LH) (pLH – Sigma Aldrich, Dorset, UK) or Follicle Stimulating hormone (FSH)(pFSH - National Hormone and Peptide Programme, Torrence, CA) at 0.0, 1.0, 10.0, 100.0ng/ml to induce testosterone production. Full details of chemical concentrations, hormone concentrations and culture medium are provided in chapter 2.11. After the 24 hour incubation, supernatants were collected and frozen (-20°C) for testosterone measures using and EIA assay.

6.2.1 Testosterone EIA Assay

Supernatant from culture wells was collected and frozen at the end of the 24 hour incubation period. Testosterone concentrations were measured in the thawed supernatants (200X dilute) using a competitive enzyme-linked immunosorbent assay (EIA) method. Supernatant was incubated on a pre coated goat/anti-mouse IgG microtiter plate (Enzo Life Science, Farmingdale, NY) and then the yellow color generated measured on a microplate reader at excitation filter 405 nm, emission filter 620 nm (Fluostar Optima microplate reader, BMG LABTECH, Ortenberg, DE). Testosterone concentrations were calculated against a standard curve of known concentrations of testosterone. Full details of testosterone EIA assay are provided in Chapter 2.12.

6.2.2 Sperm Culture with environmental chemicals

Ejaculates were collected from stud dogs (n = 7) located at the Guide Dogs for the Blind National Breeding Centre (Leamington Spa). Protocol for collections and laboratory analyses are provided in Chapter 2.4. Fertility parameters were recorded at time of collection: volume (ml), motility, sperm concentration, total sperm output, total number and live Live dead ratio assessed using a Nigrosin/Eosin Stain according to WHO (2010).

The sperm was extended (20:1) in a Phosphate Buffered Saline (PBS) ethanol solution (0.1% solvent as per Pocar, 2005) containing 4 concentration concentrations of three individual EDs and one mix of EDs (PBDE 47, PCB 153, DEHP, mix PCB 153/DEHP) dissolved in ethanol (0.1% in final solution). The concentrations of PCB 153 and DEHP incubated with sperm were 2X, 10X and 100X the mean background concentrations found in testes identified in chapter 3.3.3 (Mean PCB 153 = 0.90 µg/kg; DEHP = 0.31 90 µg/kg). The sperm rich fraction of the ejaculate

was cultured in concentrations of PBDE 47 at 2X and 10x (100X concentration was not possible with PBDE 47 for methodological reasons).

Dilution medium was not tested for background concentrations of chemicals. Therefore the control concentrations may have been less than the concentrations. This was an accepted limitation of the methodology.

Post culture, the sperm were assessed for motility using a computer assisted sperm assessment system (CASA), the Hobson Sperm Tracker (Hobson Tracking Systems Ltd, UK), as described by Smith (Smith, 2000), using settings described by Hobsons Tracking Systems Ltd. Each treatment and the control were tracked for one minute at four time points (0, 60, 120, 240 minutes). This protocol is fully described in Chapter 2.5.

The sperm motility parameters measure were straight line velocity (VSL), curvilinear velocity (VCL), average path velocity (VAP), straightness (SLR), linearity (LIN), amplitude of lateral head displacement (ALH) and beat cross frequency (BCF). Also recorded were dance mean (DMN), pause-variation of VSL (PFT), mean angular head displacement (MAD), and progressive motility (WHO, 1996). These are defined fully in Chapter 2.5.

The above methodology was repeated for each ED (PBDE 47, PCB 153, DEHP and mix PCB153/DEHP) at each concentration (2x, 10x, 100x) to provide 0 minute results for the four treatments and the control solution and then repeated in its entirety again at 60, 120 and 240 minutes.

6.2.3 DNA Fragmentation/Sperm Chromatin Dispersion test

Ejaculates were collected from stud dogs (n = 10) located at the Guide Dogs for the Blind National Breeding Centre (Leamington Spa). The collections were done and processed as describe for previous sperm cultures and full protocols can be found in Chapter 2.13. On arrival at the laboratory, samples were assessed for motility (Total motility, Rapid Progressive motility, Non motile) based on a count of 100 individual sperm. The number of live and dead sperm was assessed using a Nigrosin-Eosin (Hancock) Stain as described previously above based on a count of 200 individual sperm.

Mean background concentrations of PCB 153 and DEHP, determined by previous analysis of adult canine testis as outlined in Chapter 3.3.3 (Mean PCB 153 = 0.90 µg/kg; DEHP = 0.31 90 µg/kg), were used at 2, 10 and 100x testicular concentrations.. DNA fragmentation was done using a simple sperm chromatin dispersion and brightfield microscopy was used to count cells with and without a 'halo' indicative of non-fragmented and fragmented respectively (see Chapter 2.13 for full details).

6.2.4 Hypo-osmotic swelling test

The methodology for the Hypo-osmotic swelling test (HOS) has been described previously in the literature (England & Plummer, 1993) and the exact protocols used herein fully in Chapter 2.6

6.2.5 Statistical analysis

Statistical analysis was carried out using a general linear ANOVA model using GenStat Discovery Edition 4 statistical analysis package (VSN International) and/or GraphPad Prism 6.07 Windows, GraphPad Software, La Jolla California USA, "www.graphpad.com". The assumptions for ANOVA of independent Normally-distributed errors were confirmed by examination of Genstat's residual plots. Chemicals (PCB153, DEHP) were tested singly and in combination as a 2 chemical by 3 concentration by 4 times split plot factorial design. Raw data collected from the Hobsons Sperm Tracker was used and comparisons made using a general ANOVA model. Data was fitted to the model so that interactions between the concentration of each chemical and time could be assessed. Dogs were considered to be replicate blocks and sperm samples within dogs as the main plots. The main effect of concentration group was tested against the main-plot between-sample error; all other factors and their interactions were tested against the residual sub-plot error. Means and standard error of the difference values were plotted or expressed in the text and P-values of <0.05 were considered statistically significant.

6.3 RESULTS

6.3.1 OPTIMISATION OF THE TESTIS CULTURE PERIOD: MORPHOLOGY

The qualitative assessment of the canine testis cultured for four different incubation periods (6, 12, 24, 48h) revealed that the 24 hour culture period was optimal in terms of tissue viability and morphology (Fig 6.1C). Tissue degradation and necrosis at the 48 hour point was clearly greater than at 24 hours (Fig 6.1D vs C).

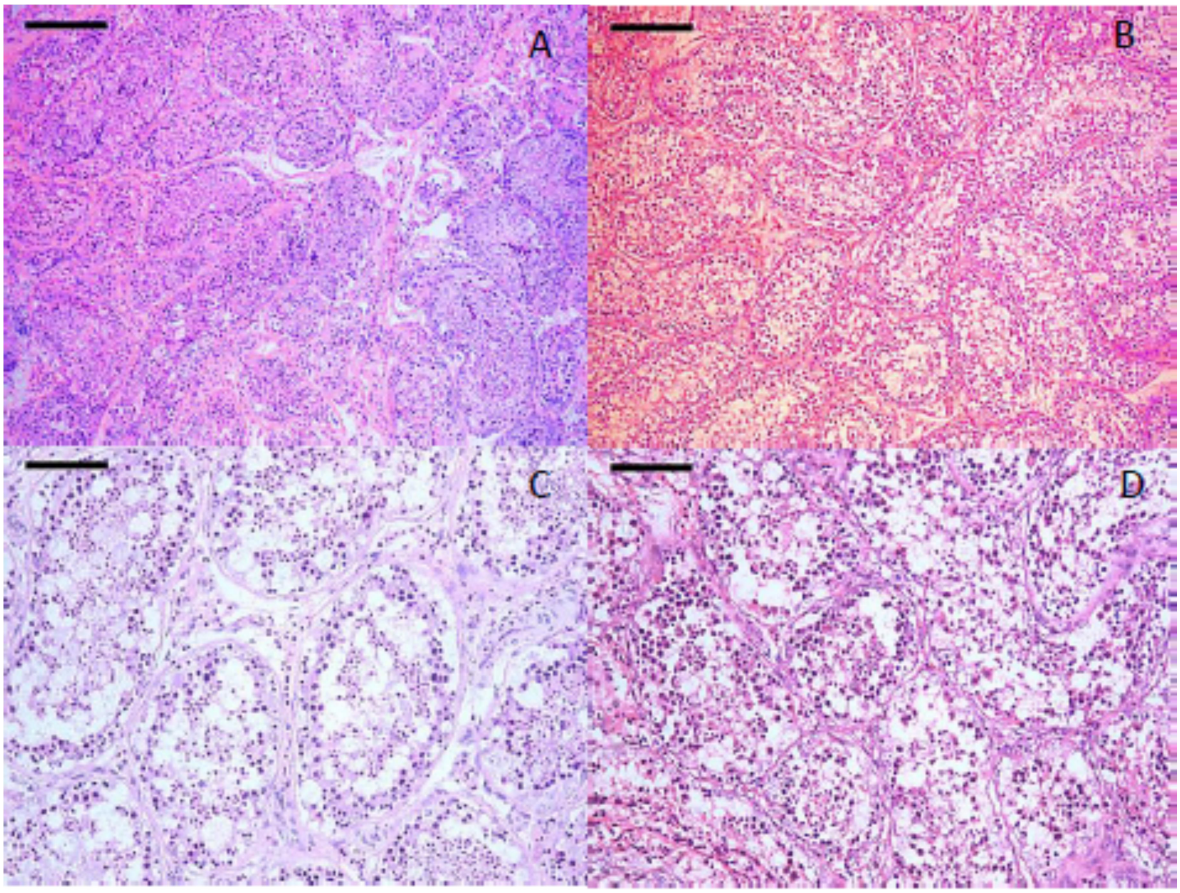


FIGURE 6-1 CANINE TESTIS EXPLANT MORPHOLOGY FOLLOWING INCUBATION IN VITRO FOR DIFFERENT PERIODS OF TIME. A) 6H, B) 12H, C) 24H, D) 48H. SCALE BAR = 100 µM. THE 0H INCUBATION TIME IS NOT SHOWN AS THERE WAS NO VISUAL DIFFERENCE BETWEEN THIS AND THE 6H INCUBATION PERIOD.

6.3.2 ESTABLISHING AND USING THE TESTOSTERONE EIA: QUALITY CONTROL AND TISSUE VALIDATION

Determination of the effect of the chemical vehicle (DMSO) on testosterone secretion *in vitro* was completed. Tissue in culture wells containing vehicle only medium showed no significant difference in testosterone production compared to the tissue cultured in the solvent vehicle (0.01% DMSO). The mean concentrations were 883.93 pg testosterone per mg of testis tissue for the wells containing vehicle and 1093.43 pg/mg testes for the medium only wells (Non Vehicle). Figure 6.2 shows the mean concentrations of testosterone cultured with and without the DMSO vehicle.

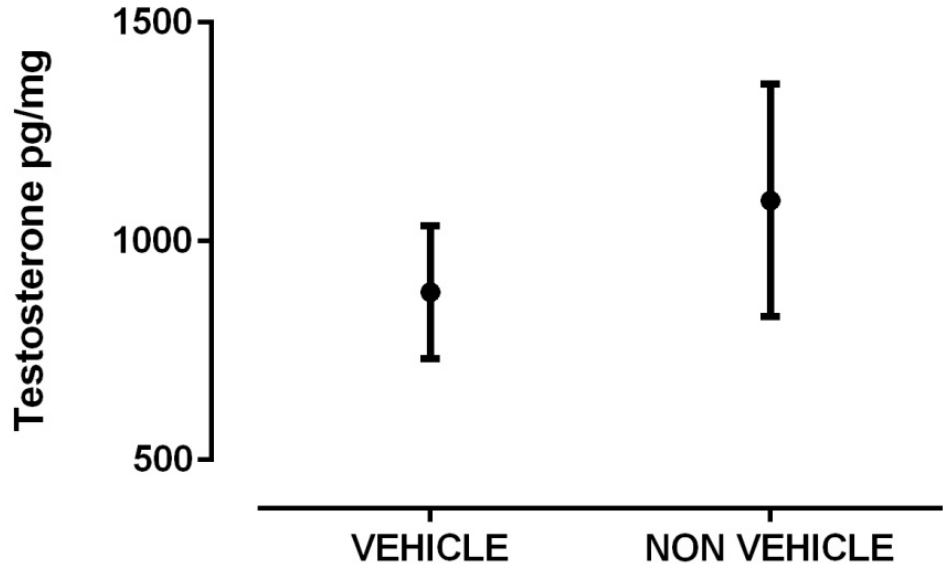


FIGURE 6-2 EFFECTS OF DMSO CHEMICAL SOLVENT ON TESTOSTERONE SECRETION IN VITRO BY CANINE TESTIS EXPLANTS: CULTURE WELLS CONTAINED VEHICLE (0.01% DMSO) OR CULTURE MEDIUM ONLY (NON VEHICLE). THERE WAS NO SIGNIFICANT DIFFERENCE IN THE MEAN CONCENTRATIONS OF TESTOSTERONE (PG/ML) MEASURED. ERROR BARS = SEM.

Establishing testosterone secretion *in vitro* using Dibutyryl cAMP was done in order to determine a physiological response and therefore tissue viability. Dibutyryl cAMP, as a cell-permeable cyclic AMP analogue, demonstrated active testosterone production in testes cultures established from 8 dogs. Figure 6.3 shows the concentrations of testosterone

measured in control wells and tissue wells cultured with Db cAMP (10 mMol/ml). Overall mean testosterone concentrations measured in control wells were 791.7 ± 128.8 pg/mg tissue with mean concentrations measured in the Db cAMP cultured wells at 3005 ± 1122 pg/mg tissue.

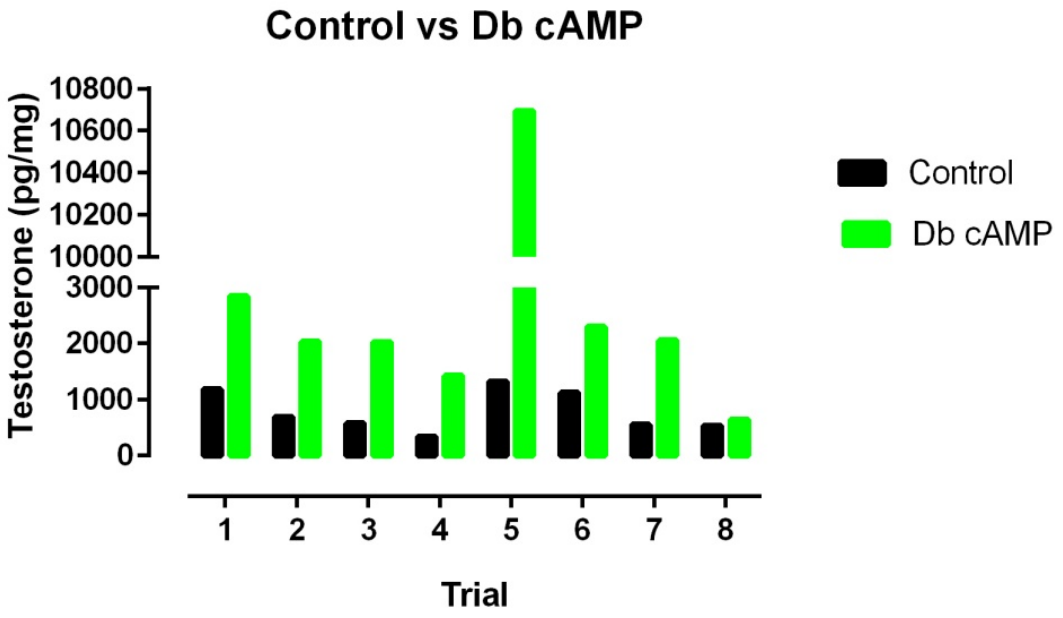


FIGURE 6-3 CONCENTRATIONS OF TESTOSTERONE MEASURED IN CANINE TESTIS EXPLANT CULTURES (PG/ML) GENERATED FROM 8 DOGS. TESTOSTERONE PRODUCED BY THE DB CAMP CULTURED TISSUE SAMPLES (GREEN) WAS CONSISTENTLY HIGHER THAN THE CONTROL (BLACK).

Testosterone secretion *in vitro* establishing a dose response to LH was achieved as a method of ensuring an expected normal physiological response from the cultured tissue samples. Testosterone secretion *in vitro* was augmented in response to increased doses of LH of 0, 1.0. 10.0, 100.0 ng/ml . Figure 6.4 shows the mean testosterone concentrations of the explant tissue culture runs (n = 8). The mean concentrations of testosterone increased in a dose response manner to concentrations of LH.

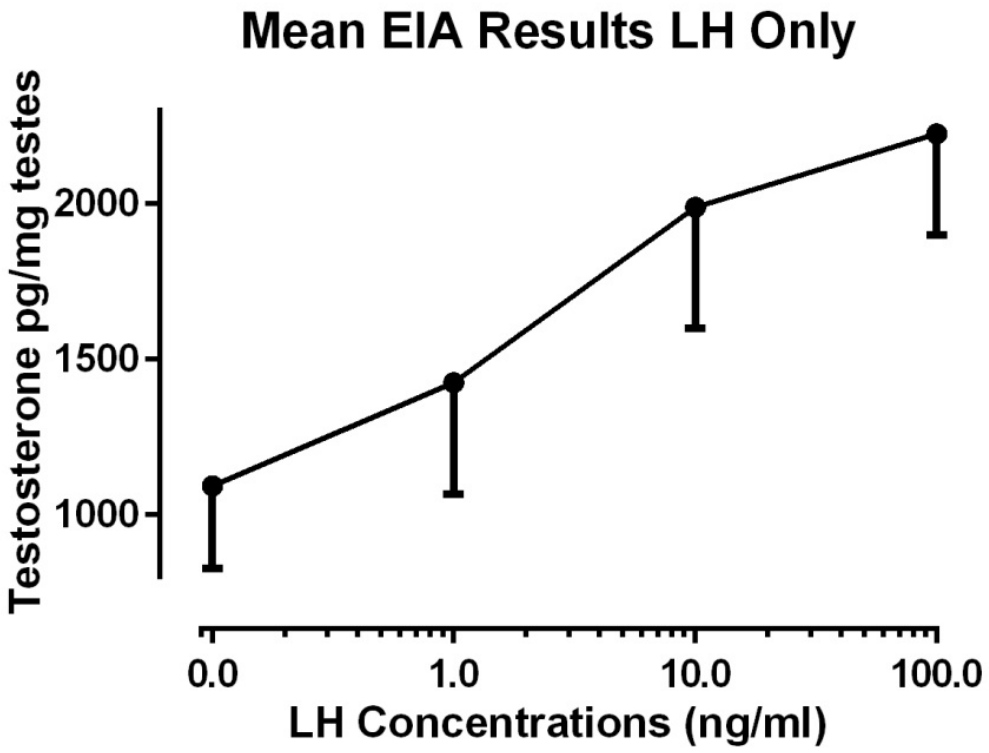


FIGURE 6-4 DOSE RESPONSE OF ADULT CANINE TESTIS EXPOSED TO INCREASING CONCENTRATIONS OF LH. RESULTS = MEAN - SEM (N=8).

A standard curve of known testosterone concentrations was created for each EIA plate. The concentration range of testosterone for the purposes of creating the standard curves was 2000, 500, 125, 31.25 and 7.81 pg/ml. The mean of all (n = 8) standard curves (± SEM) is shown in Figure 6.5. A standard curve is shown for both percent bound (B/B_O%) and net optical density (OD). The Coefficient of Variation (CV) was calculated as a measure of both intra assay (17%) and inter assay (20%) variation.

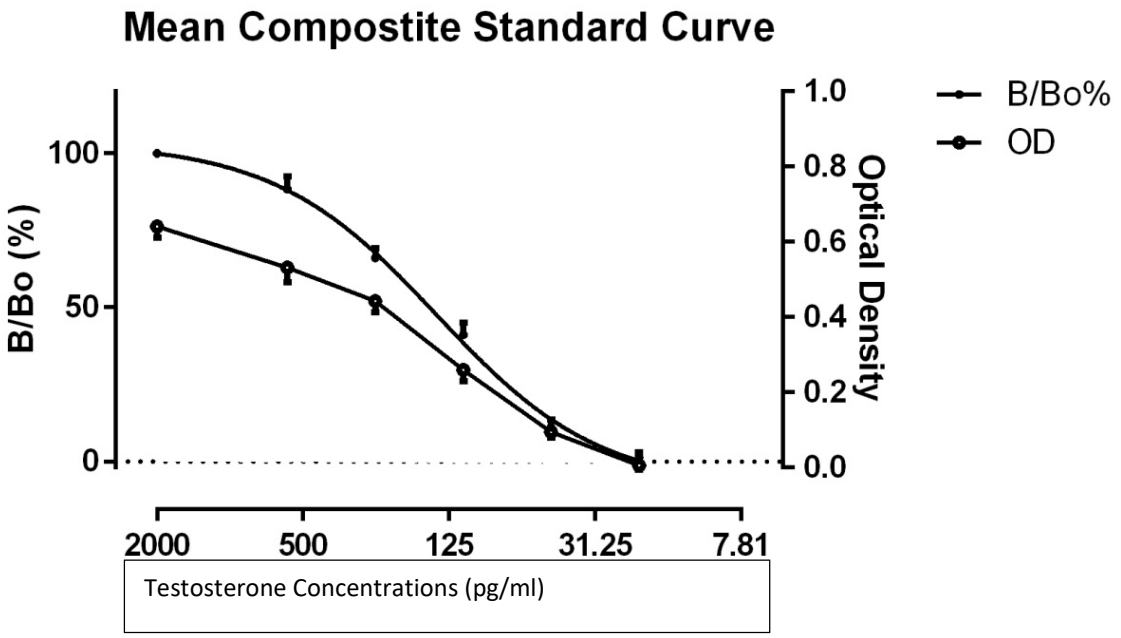


FIGURE 6-5 MEAN COMPOSITE STANDARD CURVE FOR ALL TESTOSTERONE EIA ASSAYS (N = 8). LEFT Y AXIS REPRESENTS PERCENT BOUND (B/BO%) AND RIGHT Y AXIS REPRESENTS NET OPTICAL DENSITY (OD).

6.3.3 EFFECTS OF PCB153 AND DEHP ON LH STIMULATED TESTOSTERONE SECRETION

Testis explants were incubated with LH at 1, 10 and 100 ng/ml in the presence and absence of PCB153 or DEHP. Figure 6.6 shows LH induced testosterone secretion in explant testes cultures (n = 8) when incubated with 10 and 100X mean background concentrations of PCB153. A dose response to LH was demonstrated in the presence or absence of PCB153.

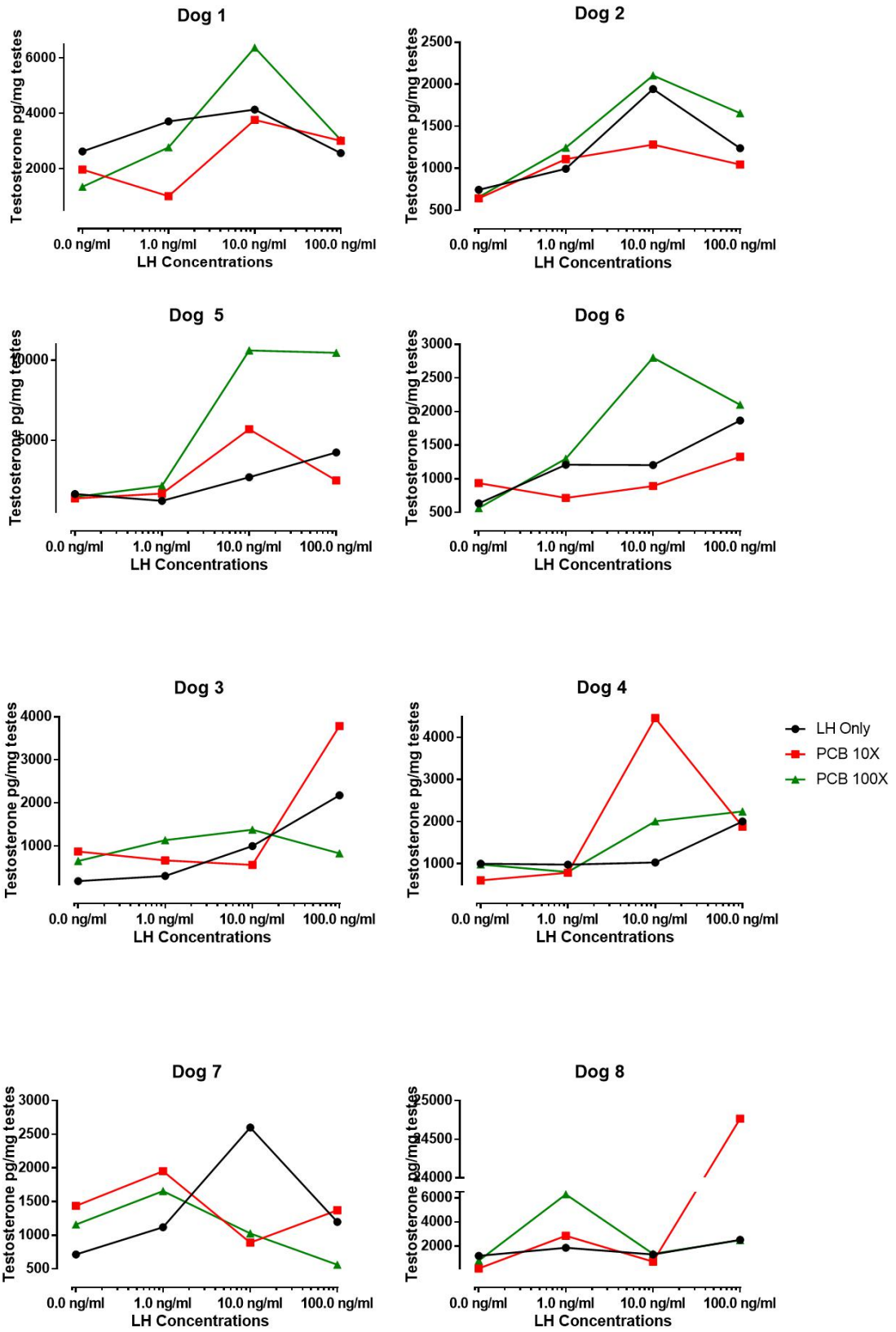


FIGURE 6-6 TESTOSTERONE CONCENTRATIONS MEASURED IN EXPLANT CULTURE MEDIA FROM TISSUE CULTURES EXPOSED TO THREE CONCENTRATIONS OF LH AND TWO CONCENTRATIONS OF PCB 153.

The effects of DEHP on LH stimulated testosterone secretion from each dog testis explant (n=8) is shown in Figure 6.7. DEHP effects are shown for 10 and 100x background (testis) concentrations.

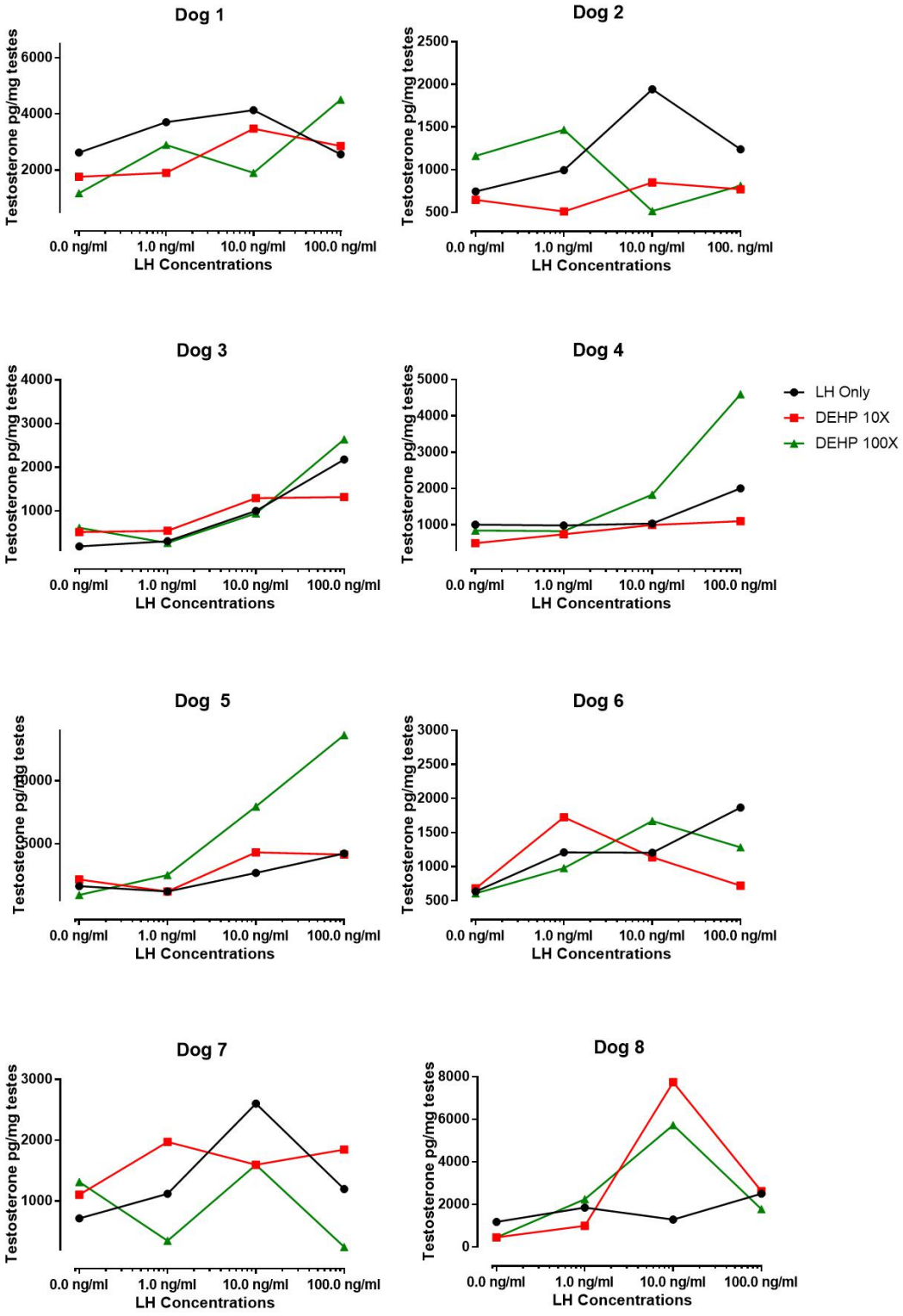
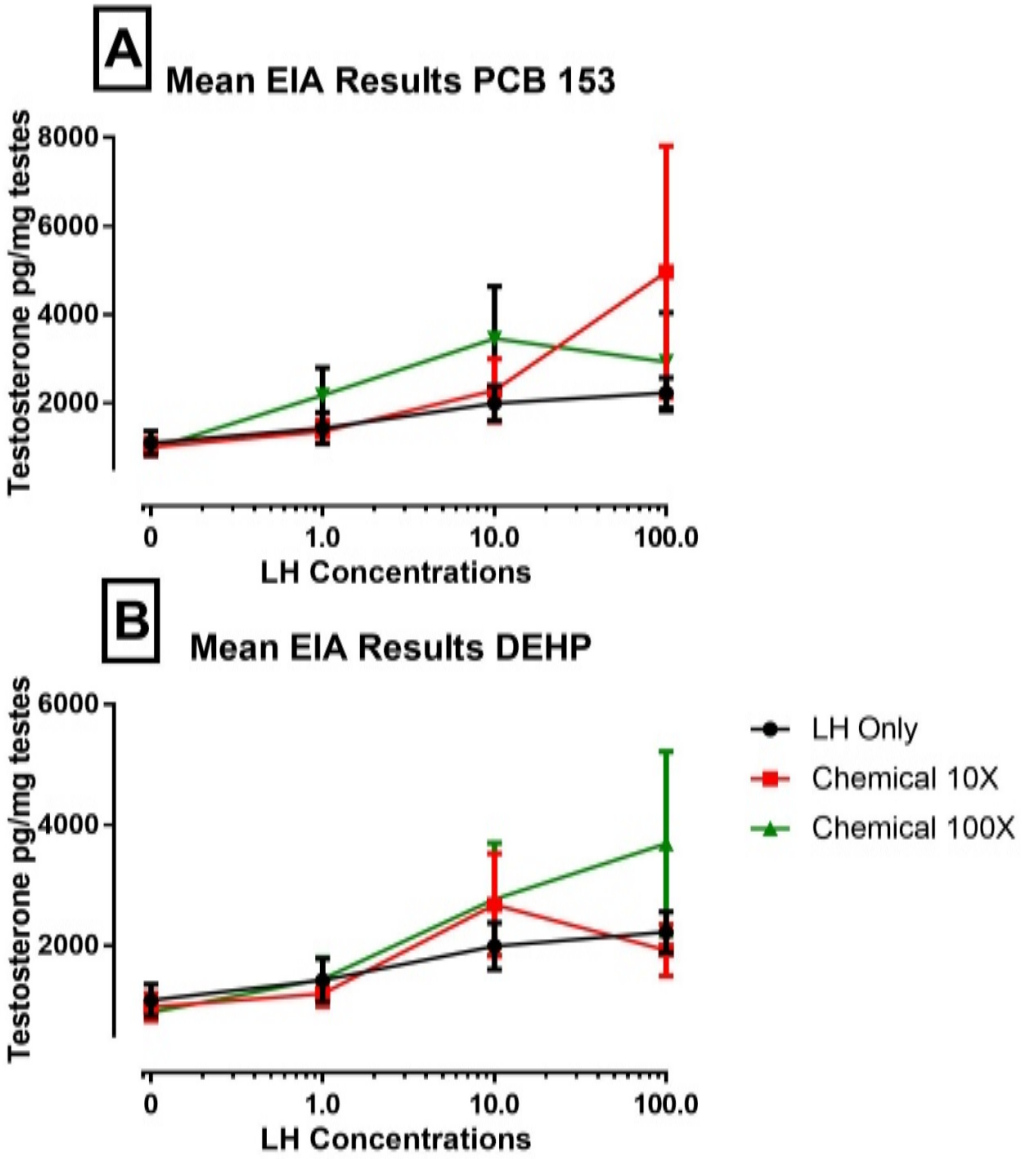


FIGURE 6-7 TESTOSTERONE CONCENTRATIONS MEASURED IN EXPLANT CULTURE MEDIA FROM TISSUE CULTURES EXPOSED TO THREE CONCENTRATIONS OF LH AND TWO CONCENTRATIONS OF DEHP.

The mean values for LH stimulated testosterone secretion (n=8) in the presence and absence of PCB153 and DEHP at 10 and 100x background testis concentrations are shown in figure 6.8.



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FIGURE 6-8 TESTOSTERONE CONCENTRATIONS MEASURED IN EXPLANT CULTURES MEDIA FOR TISSUE CULTURED WITH THREE CONCENTRAITONS OF LH AND TWO OF BOTH PCB 153 AND DEHP.

Figure 6.9 shows proportional concentrations of testosterone in relation to percent difference from the control (0.0 ng/ml LH). The bar in figure 6.9 shows the SED for the group with three concentrations of LH for each treatment group shown. The 0.0 ng/ml LH control group is omitted but represented as 100% with the dotted horizontal line. There was no statistically significant difference found in the

concentrations of testosterone produced in culture systems when tissue was exposed to EDs (PCB 153; DEHP).

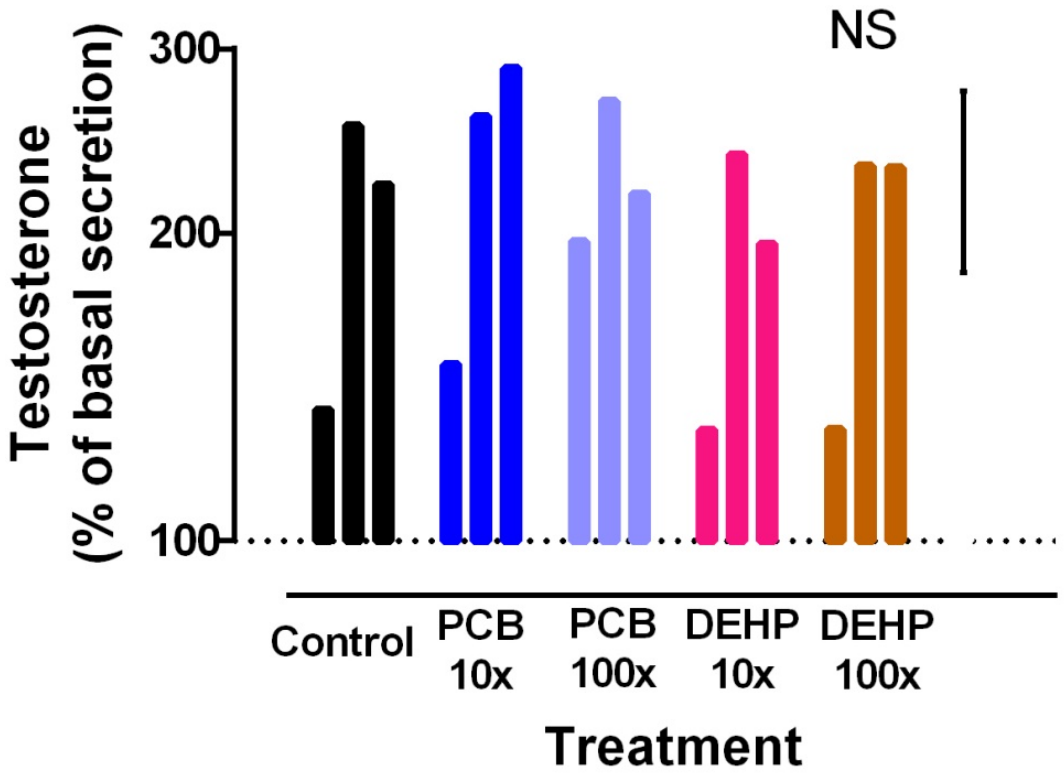


FIGURE 6-9 EFFECTS OF PCB153 AND DEHP ON LH STIMULATED TESTOSTERONE SECRETION BY CANINE TESTIS EXPLANTS. LH DOSE RESPONSES ARE SHOWN IN THE ABSENCE OF CHEMICAL (BLACK) AND IN THE PRESENCE OF EACH CHEMICAL INDEPENDENTLY. CHEMICALS WERE TESTED AT 10X AND/OR 100X THE CONCENTRATION DETECTED IN TESTIS. BAR = ±SED.

6.3.4 EFFECTS OF CHEMICALS ON PARAMETERS OF SPERM QUALITY MEASURED *IN VITRO*

The Effects of chemical vehicle (0.1% ethanol) on sperm motility measured by CASA was done to determine any confounding effect of the solvent on the overall results. In order to achieve this sperm was cultured in the presence and absence of 0.1% ethanol and subjected to the following parameters of sperm movement: straight line velocity (VSL), curvilinear velocity (VCL), average path velocity (VAP), straightness (SLR), linearity (LIN), amplitude of lateral head displacement (ALH) and beat cross frequency (BCF). Also recorded was dance mean (DMN), pause-variation of VSL (PFT), mean angular head displacement (MAD), and progressive motility. No significant differences were observed for any of the motility parameters. Therefore this solvent was deemed to have no confounding effect on the results.

Effects of PCB153 and DEHP on sperm motility measured by CASA showed significant changes in specific parameters of motility. Culturing of sperm in a medium containing testicular concentrations (2 and 10X mean testes concentrations) of PCB 153 had significant effects on specific parameters of sperm motility measured using CASA. Figure 6.10 illustrates that PCB 153, significantly increases per cent linearity (Fig 6.10A: $P< 0.05$), per cent motility (Fig 6.10B: $P< 0.05$) and Straight-line velocity (VSL) (Fig. 6.10D: $P < 0.05$). Percent straightness (Fig 6.10C) was not significantly altered but, in line with the other 3 parameters, showed a trend towards an increase.

Culturing sperm in a medium containing a phthalate ester, DEHP, had a significant effect on specific parameters of sperm motility as measured using CASA. Figure 6.10 illustrates that DEHP significantly decreases per cent linearity (Figure 6.10A: $P < 0.05$). DEHP significantly decreased per cent straightness as illustrated in Figure 6.10C ($P < 0.05$) and Figure 6.10H

illustrates that DEHP significantly decreases straight line velocity ($P < 0.05$). Figure 6.10F illustrates a non significant decrease in per cent motility when sperm was cultured with DEHP.

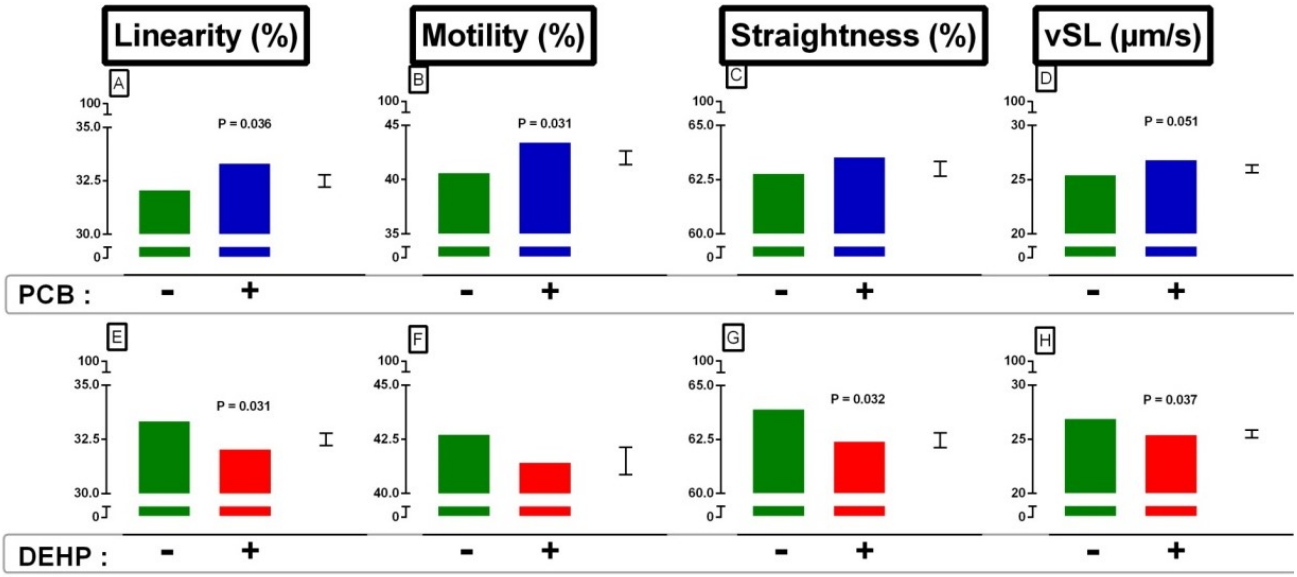


FIGURE 6-10 EFFECT OF PCB 153 AND DEHP ON FOUR MOTILITY MEASURES: PERCENT LINEARITY, PERCENT MOTILITY, PERCENT STRAIGHTNESS AND STRAIGHT LINE VELOCITY . SPERM CULTURED WITH PCB153 [A – D]: SPERM CULTURED WITH DEHP [E – H]. CONTROL CULTURES WITH NO CHEMICAL ARE INDICATED IN GREEN WITH '–' ; TREATMENT CULTURES WITH CHEMICALS ARE INDICATED IN BLUE (PCB 153) OR RED (DEHP) WITH '+'. ERROR BAR = 1 SED.

Treatment with PBDE 47 did not result in any significant changes in any of the parameters of sperm motility recorded at any concentration using the CASA system.

Effects of two chemicals (PCB153, DEHP) on sperm vitality, as measured by the hypo-osmotic swelling test, indicated significant differences. Both PCB153 (Figure 6.11A) and DEHP (Figure 6.11B) significantly reduced sperm vitality measured by the hypo osmotic swelling test (PCB153: $P<0.05$, DEHP: $P<0.01$). DEHP also had a significant effect on the HOS test between time points 0 and 240 minutes ($P < 0.05$) with a decrease in the number of cells positive to the HOS test at the second time point. Results (predicted values: genstat) are represented in Figure 6.13.

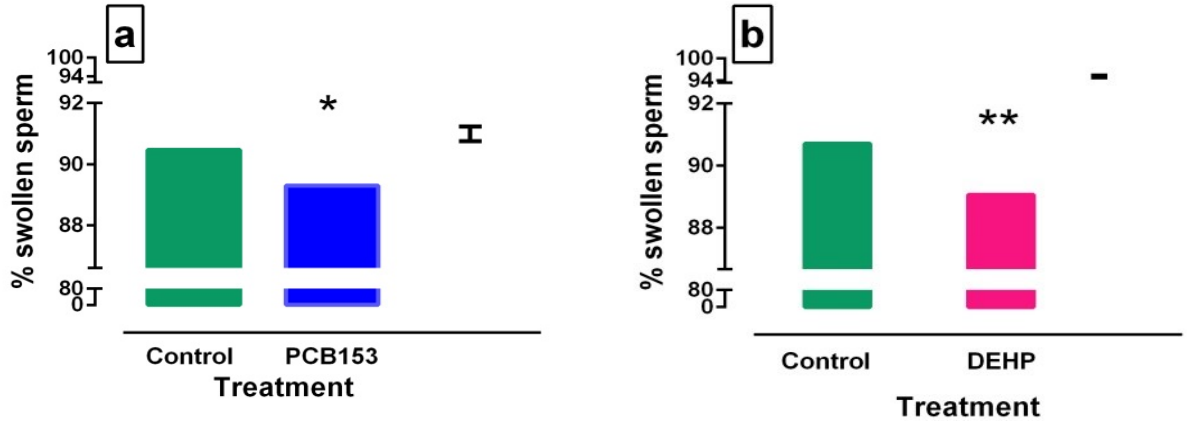


Figure 6-11 **VITALITY OF SPERM CULTURED WITH CHEMICAL POLLUTANTS. A) THE PERCENTAGE OF SPERM POSITIVE TO THE HOS ASSAY WHEN CULTURED WITH PCB153. B) THE PERCENTAGE OF SPERM POSITIVE TO THE HOS ASSAY WHEN CULTURED WITH DEHP. BAR = 1 SED.**

Sperm motility measures quantified by the CASA system and analysed by ANOVA general linear model (genstat) are summarised in Table 6.1

TABLE 6-1 THE SIGNIFICANT EFFECTS OF PCB 153 AND DEHP ON PARAMETERS OF SPERM MOTILITY (*OVERALL: INDEPENDENT OF TIME AND CONCENTRATION). ARROWS INDICATE DIRECTION OF EFFECT AGAINST CONTROL.

Sperm parameter affected	Chemical effect	Level of significance
Straight Line Velocity (VSL)	PCB (overall)* ↑	P=0.051.
	DEHP (overall)* ↓	P= 0.0037
% Linearity	PCB (overall)* ↑	P=0.036
	DEHP (overall)* ↓	P=0.031
Pause Frequency of the VSL (PFT)	PCB (overall)* ↑	P=0.004
	PCB x Time ↑	P=0.001
% Motility	PCB (overall)* ↑	P=0.031
% Straightness	DEHP (overall)* ↓	P=0.032
HOS	PCB (concentration) ↓	P=0.026
	DEHP (time) ↓	P=0.041

6.3.5 EFFECTS OF ENVIRONMENTAL CHEMICALS ON SPERM DNA FRAGMENTATION

Prior to measuring the effects of chemicals on sperm DNA fragmentation, the vehicle (0.01% DMSO) was tested against control medium only. Figure 6.12 illustrates that the vehicle does not significantly affect sperm DNA fragmentation indicating that DMSO will have no confounding effects.

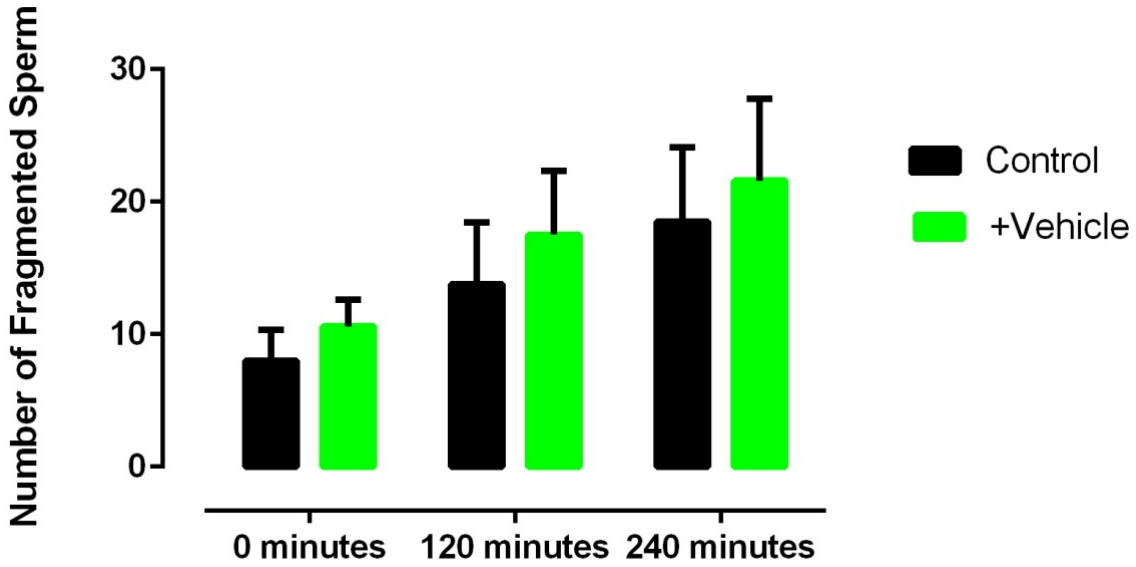


FIGURE 6-12 EFFECTS OF CHEMICAL VEHICLE ON CANINE SPERM DNA FRAGMENTATION FOLLOWING INCUBATION FOR 120 AND 240 MINUTES. NO SIGNIFICANT DIFFERENCE WAS DEMONSTRATED BETWEEN SPERM CULTURED WITH EXTENDER ONLY (CONTROL) AND EXTENDER CONTAINING 0.01% DMSO (+ VEHICLE). MEAN NUMBER OF FRAGMENTED SPERM COUNTED (N = 200) IN CULTURE TRIALS (N = 10); BAR = ± SEM.

Effects of PCB153 and DEHP on canine sperm DNA fragmentation and live:dead ratio were significant. A significant effect ($P < 0.001$) was found in the proportion of sperm fragmented when cultured in DEHP and PCB 153 for 120 minutes. This effect was seen at both chemical concentrations independent of time as shown in Figure 6.13. The analysis of the number of live sperm measured at time point 0 and 120 minutes showed a significant difference between the control group and the groups exposed to DEHP and PCB 153 ($P = 0.001$). The number of live sperm in the control and two treatment groups is shown in figure 6.16 expressed as a ratio (Live:Dead).

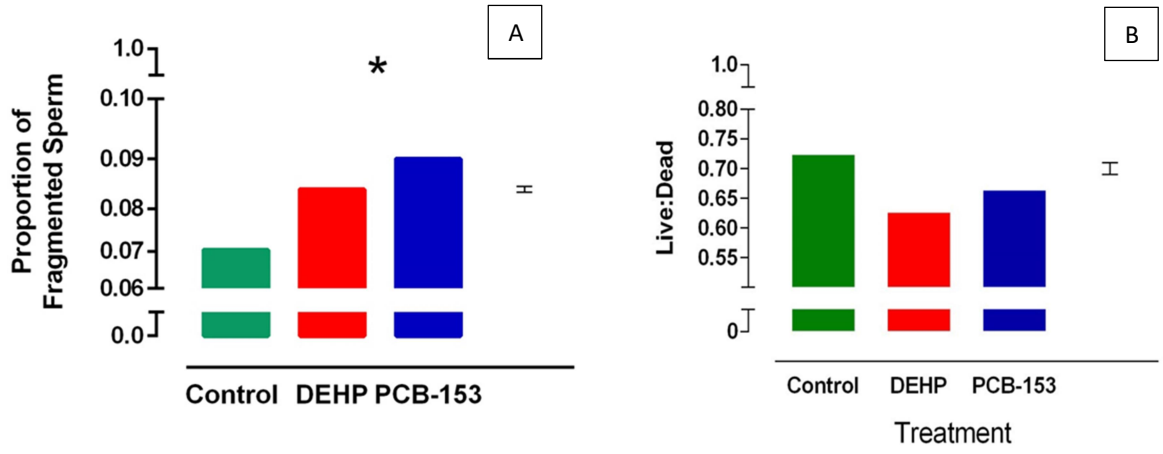


FIGURE 6-13 SPERM VITALITY AS MEASURED BY DNA FRAGMENTATIONS AND LIVE:DEAD RATIO. A)SPERM DNA FRAGMENTATION WHEN MEASURED AFTER 240 MINUTES (N=10 DOGS, CHEMICALS TESTED INDIVIDUALLY) WITH A STATISTICAL SIGNIFICANCE REPORTED (P < 0.001). B) LIVE:DEAD RATIO OF SPERM WHEN MEASURED AFTER 240 MINUTES CULTURE IN DEHP AND PCB 153 (N=10 DOGS, CHEMICALS TESTED INDIVIDUALLY) WITH A STATISTICAL SIGNIFICANCE REPORTED (P < 0.001).

6.4 DISCUSSION

Data presented in this chapter were designed to test the overarching hypothesis stating that environmental chemicals perturb indices of male fertility. Using unique *in vitro* methodologies as a means of assessing short term exposure effects, testicular concentrations of chemicals have been shown to alter sperm motility, DNA fragmentation, sperm vitality (hypo osmotic swelling test; live:dead ratios). Although these are indirect measures of fertility, these parameters are accepted methods for the assessment of sperm quality. The results suggest that exposure to environmental chemicals at low, non toxic concentrations are perturbing sperm function and vitality. Additionally, these alterations, if considered collectively, may have a compounding effect on sperm quality. The chronic effect *in vivo* may differ from the acute effects reported here but the amplification of the known environmental background concentrations used could mimic a long term chronic exposure. The concentrations which demonstrated an effect (2 and 10X tissue background) are environmentally relevant.

The aim of validating the explant culture system was achieved and proven by demonstrating that all the tissue had a normal physiological response to Db cAMP. The explant culture system was further validated in the all tissue produced measurable concentrations of testosterone and testosterone production was in a dose response manner to the increasing concentrations of LH.

When considering the results of all the explant cultures, as shown in Figures 6.6 and 6.7, there is a great deal of variability in the individual tissue responses. There were no variable controls for age, size and breed of the dog when collecting the tissue as it was collected opportunistically as residual from routine castrations. This may account for some of the variation and a consideration for future work.

The relatively high CV% in the EIA assay also needs to be considered. The intra assay %CV was 17 and the inter assay %CV was 20 which is relatively high when compared to those reported for other testosterone assays. Using a foetal human explant culture, intra and inter assay %CV's have been reported as low as 8.6 and 3.2% respectively (Fowler et al., 2007). Further refinements of methodologies and lab practices may reduce %CVs and therefore provide more credible results.

The assay was designed as effective with a range of 7.81 – 2000 pg/ml. In order to run the assay within this range the culture media collected was diluted 200 times for each tissue culture run. At this dilution rate the extreme of the testosterone concentrations would be measured at the top and bottom of the effective range, which is the middle of the standard curve. A more precise methodology would be to dilute more or less, as appropriate across the concentrations of LH so as to run the assay more closely to the middle of the effective range. This may provide more reliable results and lower CV% as the CV increases at the extremes of the assay.

Testosterone concentrations have been associated negatively with PCB exposure in humans (Goncharov et al., 2009). Human cell culture models have also demonstrated a negative effect of PCB 153 on testosterone production. The results achieved herein seemingly do not support this published work but neither do they dispute previous findings. Further repeated work using the now optimised canine explant culture protocol with and increased sample size and targeted donor tissue collection would be the next step in examining an effect between PCB exposure and steroidogenesis.

Sperm culture showed PCB 153 had a treatment effect on VSL ($P = 0.051$), % Linearity, PFT, % motility. All of these measures of sperm function showed a positive relationship between PCB 153 and motility. It cannot be determined if this effect would have an inverse correlation with

overall fertility. Further sperm function tests would give insight into the effect of the observed increase in these specific measures of motility. The sub populations of sperm identified using the CASA system need to be more fully understood. The capacitation process and the implications for the effects on different measures of motility need to be investigated prior to claims being made about the adverse effect of these chemicals on sperm function and ultimately fertility. Other methods of testing sperm function could complement motility. For example, an oocyte penetration assay, as outlined by England & Hewitt (1998) would be a useful tool for this investigation.

The conflict between the results reported in the literature showing a negative correlation between PCB and sperm motility (Bush, Bennett, & Snow, 1986; Dallinga, 2002; Hauser, Chen, Pothier, Ryan, & Altshul, 2003; Meeker et al., 2011; Richthoff et al., 2003) and our results need to be considered against the methodological differences. Since other studies focused on human sperm there may be a species effect. Also, these studies measured serum concentrations of PCBs, in either blood or semen, and looked at associations between these concentrations and sperm function. Our culturing, albeit at environmentally relevant concentrations, must be commented on as a methodological difference. Mature sperm exposed to background/environmental concentrations of PCB 153 may respond differently to those exposed at relatively high chronic concentration as done in previous human and rodent studies. The negative correlations reported elsewhere are with somatic burden concentrations of PCB rather than direct exposure through culturing. Although the short term acute exposure may prove a useful model in the absence of an alternative and the concentrations used are environmentally relevant when compared to concentrations considered toxic.

An additional consideration with the chosen methodology is the dilution of the semen as this may alter the background concentrations of the chemical pollutants for the control group. The background concentrations of these ubiquitous chemicals in the diluent were not tested due to resource limitations. This could create an artefact if the sperm for the control group was subsequently cultured in less than a 1X concentration. The effect on this potential reduced concentration in the control group cannot be determined but it also cannot be ignored that subsequent significant differences between control and treatments may not represent exactly the background concentrations as a control. Future work building on this methodology would determine concentrations of the chemical pollutants in the diluent for a more exact determination of a control concentration which is equivalent to background concentration in the sample.

PCB 153 is oestrogenic with ER binding capability (Cooke et al. 2001) and a CYP enzyme inducer (Wolff, et al., 1997). The treatment effect demonstrated could be due to the chemical acting on the oestrogen receptors found on the spermatozoa. Oestrogen receptors (both ERα and ERβ) have been identified on human spermatozoa (Solakidi, et al., 2005). This research reported that the ERβ was localised at the midpiece. If canine sperm, like human sperm, has ERβ receptors localised to this area then oestrogen is likely to be involved with motility. If PCB 153 action is mediated via the ER, as has been suggested (Fisher, 2004) then it may have an effect on motility.

DEHP had a negative effect on motility, specifically VSL, % linearity, % straightness and PFT. This would suggest a negative relationship to fertility and fecundity but this cannot be determined without further research. These results are supported by the risk report for DEHP produced by the European Commission which states that it is a substance which has 'toxicity for reproduction' (JRC Europe, 2008). Although the reproductive toxic insult is suggested to be

greatest to the developing foetus (Zhang, Jiang, & Chen, 2004) it may extend to spermatozoa. The effects of culturing human sperm with DEHP also have shown a negative association with motility, specifically velocity and linearity. Also in humans, a number of researchers have shown a negative association between serum concentrations and sperm quality (Hirai et al., 2015; Hsu et al., 2014; N Pant et al., 2011; Niraj Pant et al., 2008; Y.-X. Wang et al., 2015). The weight of evidence from the literature and our results suggest DEHP at environmental concentrations negatively affect sperm quality. The clinical relevance to these effects is difficult to determine especially as they could be argued as slight or subtle changes to overall motility. The consistency of the changes due to culturing sperm with DEHP (all changes indicating a negative effect) and the cumulative effect of these changes (four measures of motility decreasing) suggests that DEHP is detrimental to sperm motility and the clinical implications of this detriment warrant specific investigation.

The statistically significant results between sperm motility treatment and controls need to be considered against biological significance. The differences in motility between the treatments and controls, although reportedly significant, in actuality are relatively small. Figure 6-10 exemplifies this and it should be noted that some of the differences are in single digits with % linearity and % motility only in the 2% range. The significance of these changes in motility with regards to sperm function cannot be elucidated. For comparison, the work of Swan et al., (2002) reporting on the total number of motile sperm across three geographic regions showed differences orders of magnitude above what we have reported. The measurements of sperm function are only one aspect of the systemic effects on reproduction and ultimately fertility of environmental pollutants. As such these results, although difficult to quantify in terms of biological significance, do contribute to a weight of evidence of an environmental aetiology for negative temporal trends in canine and human male fertility.

Both PCB 153 and DEHP had a negative effect on the per cent of cells testing positive to the Hypo osmotic swelling test. As a test of membrane function, the relevance of this assay in the investigation of EDs may be questioned. What this does indicate is that there may be an insult to the function of spermatozoa beyond the endocrine action of these chemicals. This also demonstrates the complexities related to the investigation of adverse trends in human and canine sperm quality and EDs. The perturbation of endocrine function may be only one adverse mode of action of these substances and perhaps they should be described as "Reproductive Disrupting Chemicals" rather than the more limiting label of endocrine disrupting chemicals. As an example, one additional mechanism suggested for PCB negatively effecting sperm, outside of endocrine function, is via chromatin integrity as suggesting in the literature (Spanò et al., 2005) and our own research.

The treatment effects of DEHP and PCB 153 on DNA fragmentation, as a measure of cellular viability, are notable and provide further support to the body of literature reporting these ubiquitous environmental pollutants are toxic to reproduction and fertility. The mechanism of this effect cannot be fully elucidated through this research but it is interesting to note that the pattern of change between live dead ratio and fragmentation seemingly demonstrate an effect of these chemicals on cellular viability. The reduction in the number of live sperm and the increase in the percent of DNA fragmented sperm corroborate and lend credibility to these two assays. Although the increase in fragmentation was higher in the cells cultured with PCB 153 and the greatest change in live:dead was in in cells cultured in DEHP, both of these trends were similar and demonstrate that these chemicals are toxic to sperm cells. One report which, in addition to supporting our findings that PCB increased DNA fragmentation, suggested that concentrations of PCB affected sperm chromatin integrity following a monotonic dose response (Spanò et al., 2005). This suggests that PCB affects sperm via a

mechanism outside of the endocrine system and this environmental pollutant, as with perhaps many others, is affecting different systems via physiological mechanisms.

DNA fragmentation may be the result of normal cellular activities such as apoptosis and defects in chromatins restructuring during cell division. Due to these normal cellular processes, a background level of DNA fragmentation and positive assay results would be expected. Other causes of DNA fragmentation are exposure to environmental toxicants and oxidative damage. These two causes are likely to be the mechanisms behind the results shown in our research and other reports.

Published research supports the results found in our research that PCB153 is associated with DNA fragmentation. Using a SCDA to measure cellular viability, a positive association between serum PCB153 concentrations and DNA fragmentation has been reported (Rignell-Hydbom et al., 2004). Our results, from culturing sperm cells with PCB 153, is demonstrative of a more direct association as an *in vitro* method. The results of our research and those published elsewhere allow a substantiated conclusion that PCB153 is positively associated with DNA fragmentation.

There is considerable support for our results in the published literature with DEHP being reported as positively associated with DNA fragmentation by a number of researchers (Hauser et al., 2007; Niraj Pant et al., 2008; Huang ey al., 2011; Hsu et al., 2014). All of these reports are based on associations between serum concentration of DEHP and DNA damage. The culturing of sperm with DEHP, as a methodology, allows a more controlled and specific exposure and therefore direct conclusions between the two factors (Pant et al., 2011). Our results and those reported in the literature allow for it to be concluded with confidence that DEHP is positively associated with DNA fragmentation.

The mechanism underlying our results further emphasizes the multifactorial effect of environmental pollutants. It is unlikely that an endocrine disrupting effect is responsible for the increased DNA fragmentation seen in our and other researchers, results. This demonstrates the damaging effects of these chemicals acting via different physiological pathways. This also suggests a compounding effect of these chemicals, although it cannot be determined if this leads to a synergistic, additive or perhaps antagonistic damage to fertility.

Conclusions

The methodological challenges when investigating EDs and other environmental pollutants are substantial. The relevance of using concentrations which would be toxic (by traditional toxicological research) may not provide a true indication of what is happening with environmental concentrations. The NMDRC, as described by Van Denburg (2012), of some of these substances makes testing with traditional methodologies difficult. The supposition that concentrations as close to environmental gives the most valid results would seem credible. The concentrations of 2X, 10X and 100X have shown an effect but not a classic linear dose response. This short term acute expose may give insight into the effects of chronic exposure without approaching toxic concentrations which may be irrelevant considering the proposed mechanisms of EDs. Additionally, the concentrations we used for the testing have been proven to be significant biologically in the sheep model (Lea et al., 2016; Bellingham et al., 2012).

A weak negative correlation between testosterone production and PCB 153 concentrations (Richthoff et al., 2003) has been reported by other researchers (Kraugerud et al., 2010). Further refining of the tissue selection criteria and a more accurate assay for testosterone

concentrations may provide more reliable results which may be supportive of those previously reported.

The results of sperm culturing and tissue culturing both provide intriguing results which complement the existing body of knowledge. Further work, especially with tissue and cell culture models, will move the field forward by demonstrating the damage environmental pollutants are having on male fertility via multiple mechanisms.

Chapter 7 DISCUSSION

7.1 Overall Implications and Achievement of Aims

Evidence of a temporal decline in sperm quality and an increase in the incidence of a developmental reproductive disorder, cryptorchidism, in a population of breeding dogs are significant findings reported in this thesis. Additionally these negative temporal trends, especially as the data is free of the methodological inconsistencies inherent in meta-analytic human studies, provide credible evidence of a decreasing male fertility. A range of canine fluid and testes tissue contained measureable concentrations of exemplar anthropogenic chemicals known to perturb reproductive function. The presence of pollutants in canine tissue and varying concentrations geographically show exposures which may contribute to negative trends in sperm quality and reproductive development as well as give insight into regional differences reported in human fertility. The concentrations of these chemicals found in canine tissue have been demonstrated in this research to effect sperm quality and other indicators linked to fertility. Overall, our results provide evidence, directly for the dog of a decrease in male fertility with a possible environmental aetiology. Evidence presented in this work significantly contributes to the debate on declining male fertility and the link to an environmental causation.

The original aims established at the commencement of this research programme have been achieved in that negative trends in canine semen quality and cryptorchidism identified in this thesis inevitably add further credence to the negative temporal trends previously reported for humans. The hypothesis that, as a companion animal sharing its environment with humans, the dog is exposed to the same chemical pollutants as humans has been supported by the presence of these compounds in canine tissue and other somatic media. Specifically, the aim to determine the presence and concentration of EDs in a range of tissue/media has been

achieved by proving concentrations of PSBs, PBDEs and DEHP in testes tissue, canine milk, prostatic fluid and ejaculate.

Geographic variation in human sperm quality and other components of TDS is put forward as evidence of an underlying environmental aetiology. In our research the chemicals in canine tissue and body fluids were found at varying concentrations across geographic regions in the UK and two Scandinavian countries. In order to further support the suggestion of an environmental cause for geographical discrepancies in fertility, an additional aim of this research was to identify variation in histological analysis of canine testes. Certain histological assessments varied across geographic regions and significant associations between chemical concentrations were found which further supports an environmental aetiology for perturbations in male fertility.

The mechanisms leading to reproductive perturbations are multifactorial but culturing sperm with environmentally relevant concentrations of chemicals was shown to directly affect sperm function and vitality. Again this is strongly suggestive of an environmental aetiology for the decline in canine and feline fertility. Furthermore, the short term acute exposure on sperm is indicative of a direct effect of chemical pollutants on cellular function. Surprisingly, the same chemicals at the same concentrations were found not to blunt LH stimulated testosterone secretion. Although this may require further methodological consolidation, the possibility remains that the key effects of these chemicals on male reproductive tissue and sperm are primarily non-endocrine.

Overall this work strongly supports a canine research model as a relevant tool for fertility research, both canine and human, and conditions related to reproductive health with a suspected environmental aetiology.

7.2 Canine Research Model: Additional evidence Supporting an Overall Decline in Male Fertility.

The relatively short time 50 year period in which a decline in human semen quality and reproductive health was reported (Skakkebaek et al., 2001; Swan et al., 2002) is put forward as evidence of an environmental rather than a genetic aetiology (Skakkebaek et al., 2001). This is also supported by the results of our research and in fact the time period over which canine sperm quality showed a decline was shorter than the time period of human decline. This reported time period is insufficient for a genomic shift as even the most recent human genetic adaptions occurred over a 10 000 year period (Pritchard et al., 2010).

Although there is no firm consensus, an approximation of the human generational interval is 30 years (Fenner, 2005). The canine generational interval was reported as 3.88 years (Lewis, Abhayaratne, & Blott, 2015). This means that the period of time of 26 years for the decline in canine fertility represents more generations than the time period in the human research. This has implications if the mechanisms behind the decrease in sperm quality and increase in incidence of cryptorchidism are multi or transgenerational. An epigenetic effect of EDs has been demonstrated by other researchers (Anway, 2005; Jirtle & Skinner, 2007; Kundakovic & Champagne, 2011; Lea et al., 2016; Schwindt, 2015). These epigenetic factors, suggested to act via modification to DNA methylation (Bellingham, Fiandanese, et al., 2012), are more significant in animals with shorter generational intervals. This may be a consideration for future work using the dog which could be exploited to examine the epigenetic effects of chemical pollutants on male fertility.

Variation in measures of fertility and reproductive health across geographic regions also supports the argument that environmental factors are perturbing reproductive development and sperm quality in the dog. In humans this argument uses data from Scandinavian

countries, which show distinctive variation in semen quality and other factors related to reproductive health, as an example. The basis of this example is the discrepancy in sperm quality and other aspects of human male fertility between Denmark and Finland (Jensen, 2000; Jorgensen, 2002). For unexplained reasons, Denmark has historically reported lower sperm quality and higher incidence rates for other aspects of TDS than neighbouring Finland. The reason for two ethnolinguistically similar neighbouring countries having a reported difference in sperm quality is attributed to environmental factors (J. Richthoff, 2002; TSAREV et al., 2005) rather than lifestyle or genetics. The variation in many of the chemical concentrations and physiological measures related to fertility reported in this thesis also indicate geographic variation across these same two countries or perhaps more directly the regions from which tissue were collected (Denmark: Copenhagen area; Finland Helsinki area). Therefore the canine model supports the hypothesis of an environmental aetiology based on geographic variations in reproductive function and chemical pollutants. Certainly the argument of lifestyle variation is invalid when studying dog fertility and additionally supports and environmental rather than lifestyle cause for both canine and humans.

The fact that Denmark has been reported as having lower semen quality (Jorgensen, 2001, 2002) and a higher incidence of the other factors related to TDS (Nordkap et al., 2012) when compared to its neighbour Finland is one of the strongest arguments for an environmental aetiology driving secular trends in reproductive health. The targeting of Finland, Denmark and the UK for canine tissue collection and evaluation was deliberately done to determine if tissue histology and chemical concentrations show variation across these geographic boundaries and our current work did report geographic differences across these nations. In addition, quantification of area stained for the steroidogenic marker p450scc indicate differences in area stained between Denmark and those reported for Finland.

The underlying mechanism of action of the chemical pollutants which results in reduced fertility is undetermined. PCB is an androgen antagonist (Bonefeld-Jørgensen et al., 2001) and DEHP can alter testes development in rats by reducing testosterone concentrations (Gray et al., 2006). The anti-androgenic actions of these EDs may act collectively or perhaps even synergistically to alter testes development and subsequently adult fertility. This has certainly been discussed by other researchers many of which suggest that ED are either synergistic or antagonistic (Toppari et al., 1996; Pinto & Carvalho, 2013; Kabir, Rahman, & Rahman, 2015). Other researchers have suggested that two chemicals act collectively. PCB has been suggested to act synergistically with both mercury (Rignell-Hydbom et al., 2007) and, more interestingly, PBDE (Siddiqi, Laessig, & Reed, 2003). The supposition that chemicals act cumulatively is plausible but need further research to demonstrate conclusively. Our research is an example of the challenges this research presents, as we reported conflicting results for the effects of PCB and DEHP on sperm mobility. Together these chemicals may act antagonistically with the increase in motility reported with PCB being balanced against the decrease reported for motility by DEHP.

An alternative to measuring the effects a single chemical pollutant is using a mixture of chemicals representing combined environmental exposures (Axelstad et al., 2013). An example of this approach, the sewage sludge model (Paul et al., 2005), can contribute to the understanding of the combined effect of environmental pollutants and has demonstrated perturbations of ovine foetal testes via maternal exposure to sewage sludge treated pastures (Lea, Byers, Bellingham, Cotinot, Evans, Mandon-Pepin, Sinclair, 2014). Indeed the reality is that different research models are needed to understand this complex issues of environmental effects on reproduction, outlined in Figure 7.1. This multi-model approach, if nothing else, questions the validity of conclusions based on traditional toxicological methodologies when

determining safe exposure concentrations to these chemicals. The extension of this argument would suggest that in isolation a single research model and methodology is not sufficient. To fully understand the effects of chemical pollutants on an aspect of ill health a range of research models is required.

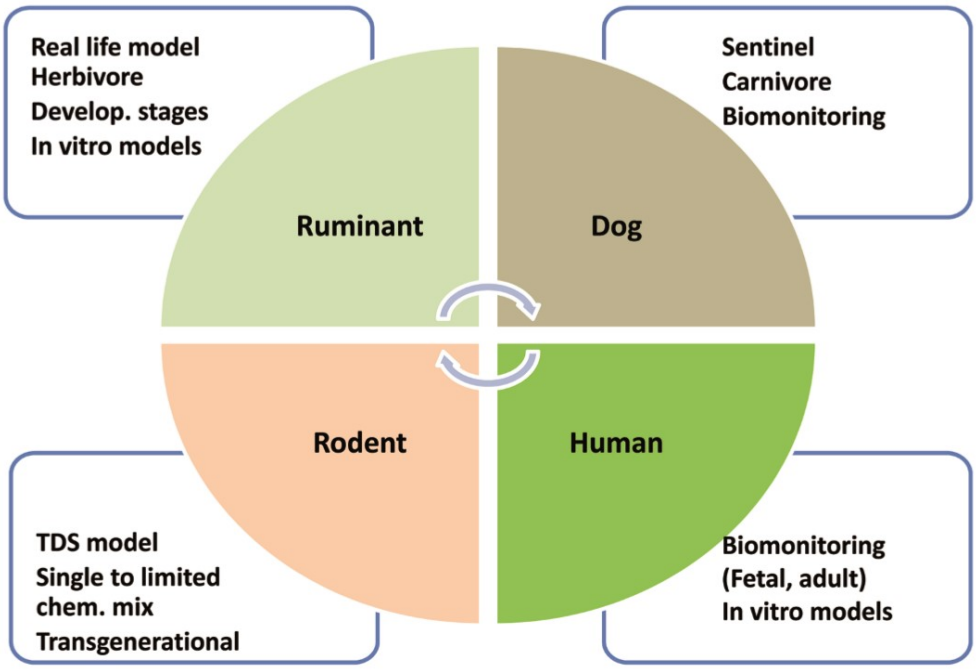


FIGURE 7-1 ROLES OF EXPERIMENTAL ANIMAL MODELS AND SENTINEL SPECIES IN DETERMINING THE REPRODUCTIVE IMPACT OF ENDOCRINE DISRUPTING CHEMICALS. RUMINANT: SHEEP EXPOSED TO A COCKTAIL OF CHEMICALS CONTAINED IN SEWAGE SLUDGE CONSTITUTE A REAL LIFE RUMINANT (HERBIVORE) MODEL OF HUMAN EXPOSURE. THE LONG PERIOD OF GESTATION AND WELL CHARACTERISED STAGES OF FETAL DEVELOPMENT FACILITATE THE ELUCIDATION OF ED EFFECTS AT SPECIFIC DEVELOPMENTAL STAGES. AN ESTABLISHED IN VITRO OVARY CULTURE SYSTEM ENABLES INDIVIDUAL CHEMICAL AND MIXTURE EFFECTS TO BE DETERMINED. RODENT: AN ESTABLISHED RODENT MODEL OF TESTICULAR DYSGENESIS SYNDROME (TDS) PROVIDES INFORMATION ON MECHANISM. THE SHORT GESTATION PERIOD HAS BEEN EXPLOITED TO INVESTIGATE TRANSGENERATIONAL EFFECTS. DOG: THE DOG IS A RECOGNISED (CARNIVORE) SENTINEL FOR HUMAN EXPOSURE TO EDS AND GONAD CHEMICAL ANALYSIS PROVIDES A MEANS OF BIOMONITORING. HUMAN: ANALYSIS OF CHEMICAL PROFILES IN ADULT AND FETAL SAMPLES/TISSUES PROVIDES A MEANS OF DIRECT BIOMONITORING AND GONAD EXPLANT CULTURE SYSTEMS ENABLE THE DIRECT EFFECTS OF EDS ON HUMAN FETAL TISSUES TO BE DETERMINED (LEA, BYERS, BELLINGHAM , COTINOT, EVANS , MANDON-PEPIN, SINCLAIR, 2014).

Our result could be considered short term acute exposure when compared to lifelong chronic exposures of humans and dogs to ubiquitous chemical pollutants. The effects of this short term acute exposure on canine spermatozoa and testes tissue to specific EDs demonstrate perturbations which are relevant to environmental exposures. These results are also indicative of the complexities of the field of research related to EDs as the experimental dose was below safe exposure limits as suggested by

NOEL (Rhind, Kyle, Kerr, Osprey, & Zhang, 2011). There are many variables to be considered in light of low and non-monotonic dose responses. With this caveat, the results reported in this research indicate that the selected EDs do have an effect on sperm function and possibly endocrine function of the testes (although statistically insignificant as reported herein). Further research with refined methodologies would be needed to state with confidence that these chemicals alter testes steroidogenesis but this could be considered pilot work for laboratory, single chemical mechanistic based investigations.

7.3 Methodological Strength of the Canine Model

The research model presented in this work, which could be termed the canine model, has certain advantages over other methodological approaches. The canine model has merit specifically when studying the effect of the environment on reproductive functions as the dog wholly shares its environment with humans and therefore the same chronic exposures. The canine model is also complementary to the laboratory rodent work and the whole mixture models as seen with sewage sludge/sheep research.

Methodological discrepancies in retrospective meta-analytic research reporting negative temporal trends in human semen quality (Pacey, 2013) are not present in the canine data and results used in our research. The methods for semen collection and assessment of canine sperm reported in this thesis have used a standardised protocol for fertility assessment providing more credible data compared to human data. Although the criticism of the retrospective human research reporting negative temporal trends in sperm quality, particularly the meta-analysis of Carleson (1992) and Swan (1996), is valid although the results should not be entirely negated by this potential artefact. The trends identified in the dog support those reported in the human as well as contribute to the overall suggestion that

environmental factors are lessening the quality of canine and human sperm as the sharing of environments between the two species supports this as a contributing factor.

Geographic variation in sperm quality, as well as the other factors which make up the human TDS, is put forward as evidence of an environmental aetiology for this syndrome. The variation between Denmark and Finland particularly has been given as evidence of an environmental aetiology (Jorgensen, 2001). In this thesis, the different geographic variation reported for the concentrations of PCB, PBDE and DEHP may support this hypothesis. The suggestions being that if environmental concentrations are reflected in the tissue concentrations reported herein, the variations in environmental ubiquity of these chemical will result in variations in their effect: i.e. sperm quality.

Denmark, with historic low reported semen quality, had a higher mean concentration of ΣPCB when compared to Finland and the UK in our results. This legacy pollutant, PCB, has persisted in the environment but is, since its ban, decreasing (Bruckmann, Hiester, Klees, & Zetzsch, 2013; Rasmussen et al., 2014) and this decreasing trend is expected to continue in Scandinavian countries (Airaksinen et al., 2014). This would suggest that if PCB is partially responsible for the decline in semen quality, via a reduction in androgens, as it inversely correlated with testosterone concentrations (Goncharov et al., 2009) then a recovery in this fertility parameter should be expected.

This is an oversimplification of a complex situation as PBDE concentrations may be increasing (Crosse, Shore, Jones, & Pereira, 2012; Law et al., 2014) with this group of chemicals being called the 21 century PCB (Siddiqi et al., 2003). Closer monitoring of concentrations of chemical pollutants in human and animal tissue and serum over time, coupled with prospective trend data on fertility, could give insight into this situation but currently this can only be speculation.

Data presented in this thesis suggest that the dog could act as a sentinel for this environmental monitoring of changes in chemical concentrations over time. Samples taken opportunistically from routine veterinary procedures could provide a benchmark of chemicals and exposures in a shared canine/human environment.

7.4 CANINE TDS

Our research has identified negative temporal trends in two of the four factors which make up human TDS, sperm quality and cryptorchidism. The two other conditions of human TDS which have yet to be evidenced in the dog are testes cancer and hypospadias. Incidence rates and overall trends in canine testes cancer are difficult to quantify as the epidemiological data, such as used for human research, is not available for the dog. In spite of this an increase in canine testes cancer has been suggested (Grieco, Riccardi, Veronesi, et al., 2008). Trends in the incidence rate of hypospadias in the dog are also difficult to quantify due to poor existing data sets. Hypospadias is not a condition often recognised in dogs as the examination required is not part of normal veterinary health check but one report, based on 15 cases visiting veterinary hospitals, suggest a 0.003% incidence rate (Hayes & Wilson, 1986) but no contemporary reports are available. So the conclusion that there is a potential canine TDS, as suggested by others (Grieco, Riccardi, Veronesi, et al., 2008) must be made with some caution but the weight of evidence is suggestive of a syndrome in the dog similar to that seen in the human.

The parallels seen in the dog, along with other considerations discussed below, may suggest a syndrome beyond TDS. It could be argued that TDS, as a term, should not include hypospadias as this is a developmental disorder of the penis not the testis. Other reproductive disorders with a suggested environmental aetiology have been reported and also would not fall under the term 'testicular'. These include human benign prostate hyperplasia (BPH), prostatitis and prostate cancers which both have all been associated with chemical pollutants (Shim, Kim, Song, & Lee, 2015). This is not surprising as one of the theories for the progression of human BPH is via perturbations to androgen concentrations (Isaacs & Coffey, 1989). As a disease of the male reproductive tract, with both a suggested

environmental aetiology and hormonal mechanism, linking this with the other four factors of TDS is not unreasonable.

Another measure which is associated with hormonal perturbations during the masculinization programming window is anogenital distance (AGD) in rats (Macleod et al., 2010) and a correlation between shortened AGD and phthalate exposure in humans (Swan et al., 2005) has been suggested. Shortened AGD has already been associated with other aspects of TDS, including sperm count (Mendiola, Stahlhut, Jørgensen, Liu, & Swan, 2011a) and has been put forward as a predictor of adult reproductive function (Eisenberg, Jensen, Walters, Skakkebaek, & Lipshultz, 2012). Anogenital distance could also be included as a factor linked with TDS due to its suggested environmental and endocrine aetiology.

The hypothesis that negative trends identified in four measures of male reproductive health and fertility have a common aetiology and therefore make up a testicular dysgenesis syndrome was first proposed in 2001 (Skakkebaek et al., 2001). Although there is credible evidence that human TDS is created out of a false paradigm (Akre & Richiardi, 2009) the syndrome and its congruent components are largely accepted. This is in spite of research reporting that cryptorchidism and sperm quality linked are not always linked to TDS (Jørgensen, Meyts, Main, & Skakkebaek, 2010). The four factors of TDS, BPH, prostate cancer all have a suggested environmental aetiology and via an endocrine mechanism. If all of these disorders have comorbidity then a syndrome beyond TDS is present. The limiting descriptor testicular dysgenesis syndrome does not sufficiently define this expanded range of disorders of sexual development and function with a suspected environmental causation. Therefore environmental pollutant induced disorders of the urogenital track (EPIDUT) are a group of disorders that occur during reproductive development or adult life which perturb the structure and/or function of the reproductive system all with and environmental aetiology.

7.5 CANINE SENTINEL MODEL

A range of species have been put forward as sentinels for the study of environmental chemicals and the subsequent ill health endpoint of these compounds. These have been reviewed previously in this thesis (see Chapter 1.14) as has an argument to validate the dog as a sentinel research model for reproductive development, health and fertility in the human male. This discussion is built around the canine as a companion and not a laboratory research animal. The results of this current research have highlighted some of the weaknesses but also strengths of the dog as a sentinel.

As with any facsimile, there are weaknesses of the canine sentinel model which are not suitable for all research methodologies. The methodology described in this thesis was designed around taking advantage of residual tissue from routine veterinary procedures, collected to determine environmental exposures of specific chemical pollutants. To determine environmental exposures the model species must be exposed to the appropriate environment. Therefore, one strength of the companion canine model is that there is no other animal which as closely shares our environment. Other companion animals do not as closely cohabit with humans as does the dog. Cats are also proposed as sentinels for environmental contamination and useful in their own capacity (Dye et al., 2007) but also usually spend considerable portions of their time budget outside and supplement their diet with hunting. Canine companions living in the house and eating a controlled diet, 100% of the dogs we tested were reported by the owners as living in the house, are from this point of view an excellent sentinel of domestic environmental exposures.

The dog is a long lived companion animals although the shorter gestation and lactation periods must be considered when assessing maternal exposures. Their longevity allows the dog to be used to identify environmental exposure. As an example, Mexican dogs living in cities with low pollution concentrations showed little or no abnormalities in their cardiac tissue when compared to their peers living in polluted areas who did show abnormalities (Calderon-Garciduenas, 2001b). These same researchers, in another publication, also reported evidence of chronic inflammation of canine lung tissue and suggested this as a warning of harm to humans, especially children, living in polluted urban

areas (Calderon-Garciduenas, 2001a). In this example the canine sentinel model provided powerful data by showing detrimental effects of pollution on another mammal living in our shared environment. It is worth mentioning, that these researchers suggest the dog as an environmental sentinel specifically for children and this is consistent with the views presented in this thesis.

The dog has also proven a sentinel for environmental exposures to the chemicals found in tabacco smoke. Cotinine is a biomarker of environmental tobacco smoke (ETS)(Benowitz, 1999) which has been reported in canine tissue at concentrations correlated with ETS exposure (Bertone-Johnson et al., 2008). This is taken as evidence that substances from ETS are passing to the dog. Also, there is no shortage of evidence that ETS exposure is resulting in ill health in the dog (Reif et al., 1992; Goldschmidt, 1998; Reif, Bruns, & Lower, 1998; Roza & Viegas, 2007). Although the dangers of both passive and active smoking are at this time indisputable, the evidence from the dog as a sentinel has contributed to the body of knowledge demonstrating the dangers of an environmental contaminant. With the luxury of hindsight it must be wondered how this data would have been viewed during the height of the tobacco debate which ran from a strong suggestion of the dangers of smoking by Benjamin rush in 1798 (Bien & Burge, 2009) to the first USA Surgeon General's recommendation against smoking in 1964.

More contemporary arguments against environmental pollutants are being supported by the canine sentinel model. Many local governments have banned (Ontario, Canada 2008) or are phasing out the use of cosmetic lawn pesticides. Pesticide use has been associated with specific cancers due to occupational exposure in humans (Koutros et al., 2015). Similarly in dogs, 2,4-dichlorophenoxyacetic acid, has been associated with canine malignant lymphoma based on their owners use as a cosmetic lawn pesticide (Zahm & Blair, 1992). Other research has linked lawn pesticides with canine bladder cancer (Knapp et al., 2014). The results of this research have led to concerns for children's exposure to lawn chemicals and the banning in many communities of the use of aesthetic lawn chemicals.

Real life exposures to environmental pollutants and ill health prove the canine research model and the dog a sentinel of environmental pollutant exposures. This is exemplified by the proximity of dogs to

flooring surfaces and household fabrics, as well as mouthing behaviour, being similar to those of children. Exposure of children, through handling and mouthing objects, to environmental chemicals was a factor removing lead from paints and toys (Markowitz, 2016). Currently, there is concern for children's health due to exposures from toys and other object to chemicals like PBDE (Hoffman, Webster, Sjödin, & Stapleton, 2016; Ionas et al., 2016) and DEHP (Lioy et al., 2015; Xie, Wu, Little, & Marr, 2016) specifically. Research using dogs has demonstrated that, like children, mouthing of toys and training devices can lead to phthalate exposure (Wooten & Smith, 2013) and dogs living in homes with plastic flooring have higher phthalate concentrations (Pålsson, 2016). The implication from this type of research for dogs directly, as well as human children indirectly, seems obvious and provides additional support for research using a canine sentinel.

7.6 Evidence Based Medicine: Whole Health

To broaden and bolster the points made regarding the canine sentinel model, environmental disease, whether due to climate change or pollutions, cannot be considered exclusively from the point of view of speciesism. The whole health model of Calvin Schwabe (1927 – 2006) puts forward the concept that wellbeing of humans, wildlife, production and companion animals should not be considered in isolation. A holistic approach with cross discipline collaboration, referred to as One Health, an extension of the whole health concept, suggests that the cross discipline study involving multiple species can give insight into the wellbeing of each within a shared environment or ecosystem.

The One Health concept, embracing an integrated approach to animal and human health has arisen out of emerging zoonotic disease with severe acute respiratory syndrome (SARS) and avian influenza disease as known examples. The focus of this concept is the integration of animal, human and environmental/ecosystem health investigations (Mazet et al., 2009). These integrated concepts are

shown in Figure 7.2 and the peripheral contexts of globalisation, cultural (lifestyle) and economics are easily linked to research into male fertility.

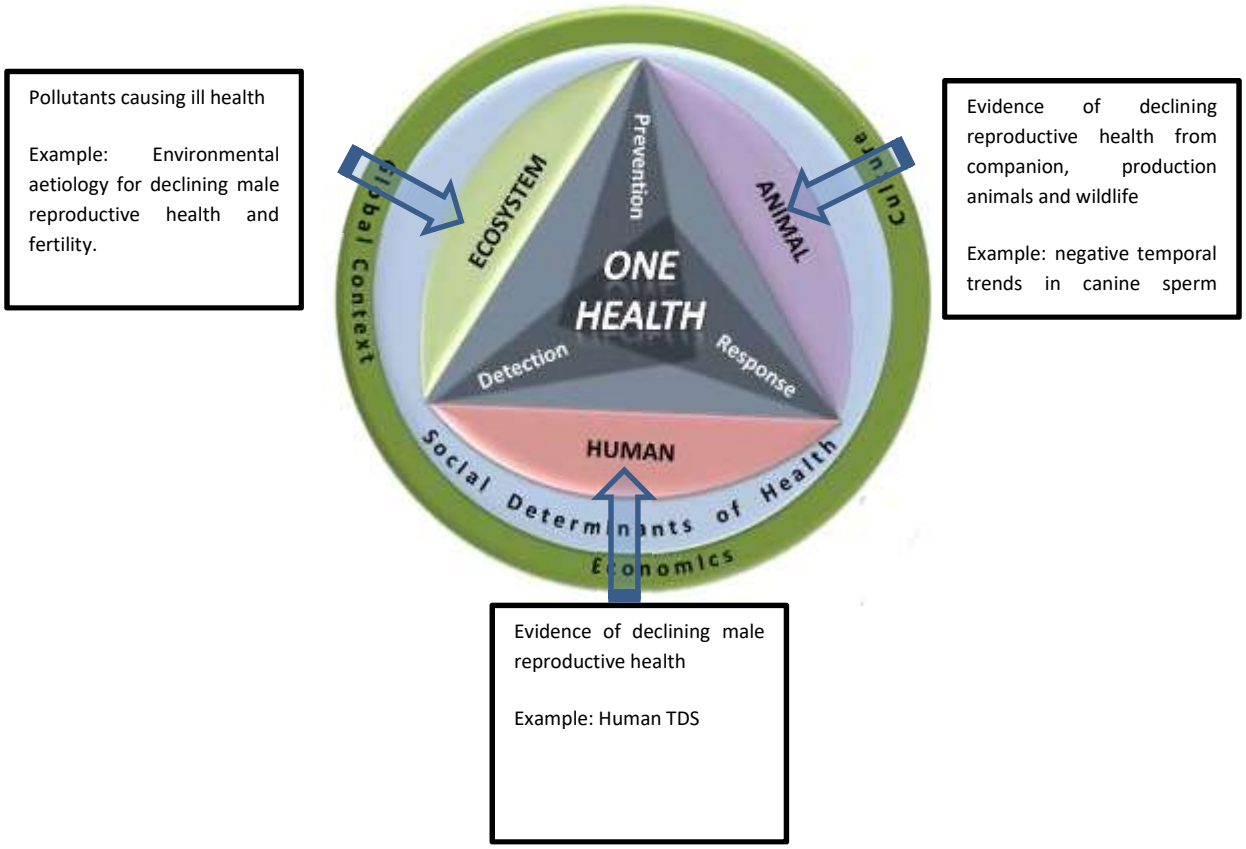


FIGURE 7-2 ONE HEALTH GRAPHIC (PUBLIC HEALTH AGENCY OF CANADA). THREE ASPECTS OF ONE HEALTH: ECOSYSTEM, ANIMAL, HUMAN WITH EXAMPLES OF EACH WITH EVIDENCES FROM RESEARCH IN MALE REPRODUCTIVE HEALTH AND FERTILITY.

The One Health approach when applied to understanding declining male fertility involves all three of the fundamental concepts. From the 'Animal' category wildlife and companion animal data showing association between adverse fertility and environmental contamination contributes to this model. Additionally, laboratory species contribute understanding of the mechanism of these chemicals. Strong associations from epidemiological human data can be made between environmental pollutants and negative temporal trends in fertility and reproductive development. From the environment, or 'Ecosystem', evidence of environmental pollutants in air, household dust, aquatic systems and soil

contributes to the model. When considered together all of these approaches to this research problem create a paradigm which is much more credible than a singular approach.

7.7 Fertility as a Biomarker for Health and Longevity

Male fertility has been linked to longevity and has therefore been suggested as a measure of overall health (Jensen, Jacobsen, Christensen, Nielsen, & Bostofte, 2009). As a holistic approach to determine health, wellbeing and longevity, fertility assessment could contribute along with other biomarkers such as blood biochemistry, blood pressure and body mass indexing. The work of Jensen et al. (2009) indicated that sperm concentration measures above 40 X 10^6 /ml were indicative of decreased mortality. This work is supported by research done on men visiting American fertility clinics (Eisenberg et al., 2014). In addition to finding associations between sperm concentrations and mortality, these researchers suggest a man with abnormal values of two or more measures of semen quality (low semen volume, sperm concentration, sperm motility, total sperm count, total motile sperm count) had increased mortality. Therefore it could be argued that men undergoing fertility assessment, especially those reporting sperm quality below a determined threshold, should have the implications for longevity explained.

Consequently, a growing body of research is suggesting a link between health, mortality and fertility (Louis, 2014; Salonia et al., 2009; Ventimiglia et al., 2015; Eisenberg et al., 2015). It seems intuitive that overall poor health will have a systemic effect and the hypothesis that reduced reproductive function is associated with ill health and mortality is acceptable. So a holistic approach to assessing health would include semen quality is proposed. This would extend to canine health and the use of the dog as a biomarker in its own right. Based on results presented in this thesis, it is proposed that continued and widespread canine fertility assessment, either semen quality or histological measures of testes tissue,

could provide data to identify temporal and geographic trends. This information could be used to support epidemiological and other research suggesting negative trends in human male fertility and reproductive function.

The mechanism linking fertility and mortality may be via testosterone concentrations. Low testosterone may contribute to lower sperm quality and other diseases linked to low testosterone may have a known comorbidity. Low testosterone has been linked to diabetes mellitus (Spark, 2007), obesity (Khaw et al., 2007), cardiac disease (Wang et al., 2011). Another biomarker associated with general health and longevity, hand-grip strength (Giampaoli, 1999), has been linked to prenatal testosterone exposure (Fink et al., 2006). The conclusion could be that all the above are indirect measures of health and longevity and testosterone concentrations is a more direct biomarker for health. Therefore, the dangers to health of any chemical pollutant which interfere with normal endocrine function during foetal development and postnatal life needs further consideration for overall health and longevity. Additionally, as identified in this thesis, chemical pollutants may have mechanisms resulting in ill health beyond the endocrine system which needs further consideration as well. Fertility as a biomarker for general health and longevity extends beyond the individual. If negative trends such as those suggested for human sperm quality are accepted then these may suggest implications for populations.

7.8 Implications Beyond Fertility

There are implications beyond fertility of the concentrations of chemical pollutants in canine tissue for dogs directly and humans indirectly. The number of human diseases linked to environmental pollutants are too numerous to mention but some key examples make the case. A group of endocrine disrupting chemicals has been linked to obesity (Newbold et al., 2008) in a subcategory of EDs labelled

obesogens (Decherf & Demeneix, 2011; Sharpe & Drake, 2013) acting via the HPG axis. The comorbidity factors with obesity are well known for humans and this disease has been called an epidemic for both dogs (Sanderson, 2012) and humans (Guthman, 2016). This has been identified as an example of a One Heath approach used to better understand a specific disease or syndrome (Sandøe et al., 2014). Obesity is an excellent example of a disease with an environmental aetiology which the canine research model can contribute to the growing body of knowledge.

Additional examples suggest a link between environmental pollutants, specifically EDs, and cancer (Soto & Sonnenschein, 2010). As well Alzheimer's disease, as normal hormonal function is necessary for cognitive function, is not surprisingly been linked to environmental pollutant which perturb hormonal prcesses (Weiss, 2007). Behavioural aberrations of children such as attention deficit hyperactivity disorder have been associated with EDs (Bouchard et al., 2010). PCB153 has a suggested developmental effect on rat brains when exposed during gestation and lactation (Hussain et al., 2000).

With numerous examples suggesting a relationship between pollution and ill health, it is not surprising that the World Health Organisation announced in 2006 that almost 24% of all diseases have an environmental aetiology. The presence in canine tissue and fluid of specific ubiquitous chemical pollutants reported in our work suggest that dogs are exposed similar to humans, so opportunities to exploit the dog as a research model extend beyond fertility. Many chemical pollutants have more than one ill health endpoint. As an example, PCB is a known endocrine disruptor (Petro et al., 2010; Spanò et al., 2005) but better known as a carcinogen (Cancer, 1987) and the mechanism of action causing cancer may be different to those causing endocrine disruption. Therefore the study of environmental pollutants is complex and a holistic approach using a range of research models is required.

It can be concluded that simplified, single model methodologies will not fully explain the link between ill health and environmental pollutants. It should be suggested that single chemicals have multiple system effects, as do the combinations of chemical found in the environment. This may be demonstrated by the effects on motility of sperm cultured with PCB and DEHP. The mechanism of this perturbation is not via the endocrine system as it is classically understood. To elucidate the action of

these chemicals by strictly focusing on endocrine action seems limiting. The evidence of disruption to the endocrine system is too weighty to ignore but the impact on the whole organism needs contemplation.

7.9 Limitations and Future Work

There are methodological strengths of our research, some of which have been mentioned previously. The dog as an environmental harbinger or sentinel is one of the main strengths which can be exploited for continued research. Others researchers have identified the dog as a sentinel model for environmental contaminants , especially for children (Enriquez et al., 2009). This point is made again in this work as the dog is exposed in a similar way to humans to environmental pollutants at relevant concentrations in a whole mixture manner. Therefore the dog is the best model for investigations designed to determine effects of real life, environmental exposures of chemical pollutants on children. An additional consideration for future work is the emotive nature of this research. The extent of anthropomorphism is greater for dogs than any other research animals. This coupled with the direct implications of environmental research on human ill health means these two factors combined may results in an increased profile of research beyond what may be achieved using laboratory rodents, for example. Linked to the point above, the ethical collection of tissue and other samples for testing used in this research can be exploited further as this model may be easier to access approval and funding specifically as surgical neutering is normal veterinary practice.

Limitations in resources meant only three types of chemical were tested in the methodologies described. The three chemical groups, PCB, PBDE and DEHP are excellent representative of

EDs and environmental pollutants but further chemicals could be used in future work. The sperm and tissue culture protocols have been established and would be easily adaptable for an expanded range of chemicals. Given the broad spectrum of actions of environmental chemicals, new and emerging chemical groups with both endocrine and non-endocrine effects should be evaluated. Other plasticisers, such as the bisphenol group are of concern as endocrine disruptors. The harmful effects of the bisphenol, BPA are well reviewed (Nachman et al., 2014) and this chemical has been removed from many plastic products. There is now concern over the replacements, specifically BPF and BPS, which may be as harmful as their predecessors (Rochester & Bolden, 2015). These are excellent examples of chemicals increasing in use and our models could be used to assess the presence in the dog, as a reflection of human exposures. Additionally, the established culture systems, sperm and explant, could be used to determine mechanistically the impacts on fertility.

Other methods of investigating mechanism of action could be employed. Cell lines are an alternative to explant cultures and these have yet to be established using canine testes cells. Methods for culturing Leydig cell lines (Toni et al., 2014), Sertoli cell lines and spermatogonial germ cell lines (Wang et al., 2016) are already established and could be adopted for canine cells.

Sperm could also be assessed with an expanded range of assays post culturing with additional chemicals. These could include capacitation status, acrosomal status, zona pellucida binding assay, oocyte penetration assay. All of these are functional assays and more directed measures of fertilisation capacity. Depending on the specific research question, these assays may more directly indicate the mechanism by which chemicals reduce fertility.

In order to more fully understand the complexity of the effect of EDs on canine fertility, future work needs to include repetition and refinement of these experiments. This needs to be

repeated for validation purposes with another group of dogs, increasing sample size and across further geographic regions.

7.10 Increasing awareness of the Dangers of Environmental Chemicals

Reports of the dangers of environmental pollutants are increasingly gaining the attentions of the general public. The recent decline in honey bee populations due to pesticide use is a recurring story in the news media. This led in part to the ban on neonicotinoid pesticides in Europe and investigations directly into the dangers of these chemicals to honey bees (Kessler et al., 2015; Rundlöf et al., 2015). More recently, research reporting the effects on the developing ovine foetus of exposure to environmentally relevant, real life, concentrations of whole mixtures of chemical pollutants via sewage sludge (Lea et al., 2016) garnered tremendous interest in the lay press. A recent headline in Time magazine state, 'That Plastic Container You Microwave In Could Be Super-Toxic' (May 14, 2016) based on research reporting the dangers of plasticisers (Attina & Trasande, 2015).

Lay press reports based on research findings are creating a shift in the attitudes of consumers and therefore a change in business practices. A joint report of several health and environmental organisations (Breast Cancer Fund, Campaign for Healthier Solutions, Clean Production Action, Ecology Center, Mind the Store Campaign) tested and found BPA in tinned food. Due to this report and other pressures, the multinational food corporation Campbell's has committed to eliminating BPA from their food packaging by 2017.

Awareness needs to be raised in the public especially pregnant women. The embryo and foetus are more vulnerable to these pollutants, especially via the endocrine system. It is now largely accepted that pregnant women should not expose their foetus to cigarette smoke and alcohol and therefore this should be extended to include known chemicals with "reprotoxic" actions. Research can demonstrate the effects of chemical pollutants but ameliorating the effects of these will come and pressure placed on the chemical and plastic manufacturers by consumers and the general public.

To complement our results, work is needed into the foetal origins of adult disease in the dog. This is especially pertinent as currently EDs are being shown to represent a serious Public Health issue and associations between environmental exposures to EDs and semen quality are being made by researchers (Le Moal et al., 2014a). Additionally, this is a biomarker for longevity and therefore mortality. These associations do not prove causation but evidence in mounting to suggest that ubiquitous environmental pollutants are causing ill health in animals and humans. Figure 7.3 shows two unrelated data sets but for the purposes of discussion present an interesting point. The is no direct suggestion of a correlation between US chemical production and the trends in US cancer deaths over a similar time period but considering that associations made between specific cancers and individual chemicals are too numerous to mention, the cumulative effect of these must be questioned.

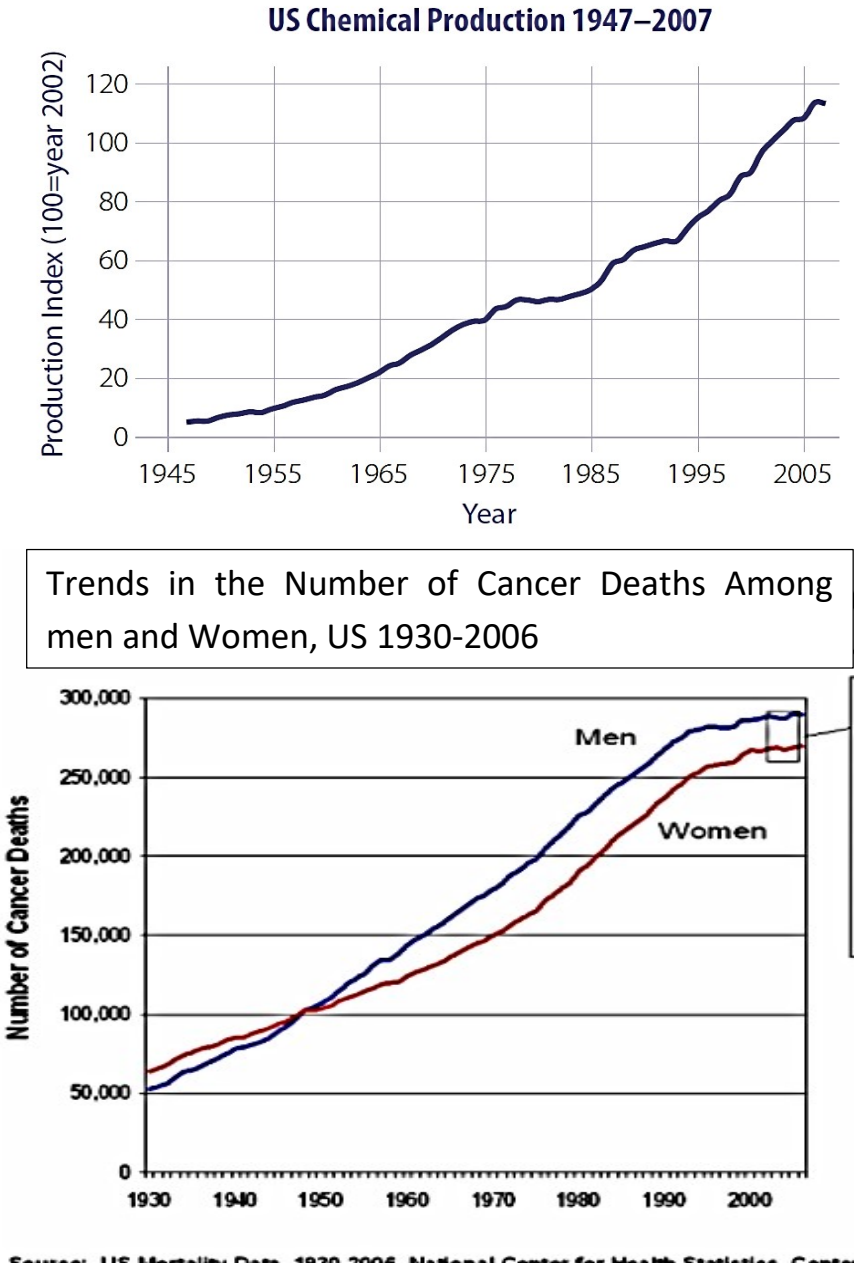


FIGURE 7-3 US CHEMICAL PRODUCTION 1947 – 2007 - SCHWARTZ, J.M. AND WOODRUFF, T.J., 2008. SHAPING OUR LEGACY: REPRODUCTIVE HEALTH AND THE ENVIRONMENT—A REPORT ON THE SUMMIT ON ENVIRONMENTAL CHALLENGES TO REPRODUCTIVE HEALTH AND FERTILITY. UNIVERSITY OF CALIFORNIA, SAN FRANCISCO, SAN FRANCISCO TRENDS IN THE NUMBER OF CANCER DEATHS AMONG MEN AND WOMEN. – NATIONAL CENTER FOR HEALTH STATISTICS, 2008. US MORTALITY DATA 1930-2006, CENTRE FOR DISEASE CONTROL AND PREVENTION

Fundamentally only two factors can contribute to fertility: genotype and phenotype. Both of these are under environmental influence, especially when epigenetics is considered a combination of genotype influence by lifestyle and our environment. An individual's environmental influences begin in the female reproductive tract from the point of fertilization

forward. A lifetime of chronic exposure to ambient chemicals has an effect on health including fertility proving a complex syndrome like TDS has an aetiology which is multifactorial. Environmental pollutants may be a chemical time bomb and the ticking is being ignored.

7.11 CONCLUSIONS

The work that has been conducted and outlined in this thesis indicates that ubiquitous, anthropogenic, environmental chemicals are impacting canine fertility and reproductive development. Endocrine disrupting chemicals have been proven to be present in canine tissue and diet. Certain EDs have been demonstrated to have an effect on sperm function, viability and motility. A direct mechanistic link between somatic burden, background tissue concentrations and reproductive function needs further investigation but our results demonstrate the need for this further work, specifically using the dog as a research model. A more direct cause and effect relationship could be further explored using a broader range of models and methodologies such as cell culture lines and sperm function assays. The strength of an historic benchmark and retrospective analysis is demonstrated and the merit of creating a resource for the collection and storage of this type of data prospectively proven.

Two overall problems are addressed by this thesis. The first addressed a lack of corroborative evidence and criticism of methodological weakness, stemming from overreliance on retrospective and meta-analytical research, for the reported temporal decline in human sperm quality. The second problem is providing evidence for an environmental aetiology for this decline. Towards a solution to these problems, a significant contribution to the field has been made by demonstrating parallel negative temporal trends in canine fertility and additional aetiological evidence using this canine model. Specific key contributions are as follows:

1. Robust, determinative evidence of negative trends in canine sperm quality and cryptorchidism in a population of dogs over a 26 year period which parallels reports in humans,
2. Specific and substantial evidence of an environmental aetiology for declining canine fertility provided by
    i. demonstrating the presence of specific exemplar chemical pollutants (PCBs, PBDEs, DEHP) in the dog's diet, testes tissue, prostatic fluid, bitches milk, ejaculate,
    ii. demonstrating geographic (intra and international) and land use variations in the concentration of these same exemplar chemical pollutants,
    iii. identifying associations between testes histomorphometric scores and concentrations of these same exemplar chemical pollutants,
    iv. demonstration of significant variation in a marker for steroidogenesis across testes tissues collected from three international regions (CYP11A1: area stained),
3. Direct mechanistic effects on canine reproductive function of exemplar chemical pollutants (PCB153, DEHP) were demonstrated by
    i. significantly perturbing sperm motility via culture with environmentally relevant concentrations of PCB153 and DEHP,
    ii. determining significant associations between tissue concentrations of DEHP and testes area stained for steroidogenic enzyme CYP11A1,
    iii. Inhibiting sperm viability as measured by hypo osmotic swelling, DNA chromatin integrity (fragmentation) and live:dead ratio assays, via culturing with environmentally relevant concentrations of PCB153, DEHP.

In summary, this work presents credible data, free of the methodological criticism of previous, historic human research, confirming a decline in male fertility. It also validates the dog as a unique sentinel and experimental model to investigate an environmental aetiology in declining male fertility. Decidedly, there can be no better human environmental proxy than our closest companion. There are weaknesses inherent in associative research, which cannot prove causation, and this should be considered in future work. Therefore additional work, as a complement to the real life, full environmental, exposure model (which the collected testes in this work represents), will come from mechanistic, experimental models as piloted in the final results chapter. Complex mechanisms of action which exposure to an environmental cocktail of chemicals have on an entire organism, and considering a specific reproductive endpoint, can only be elucidated using a range of research methodologies and models, the canine model being one of this range.

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Appendices

Informed Consent & Confidentiality Dogs Name:

Canine health and fertility research participation information

Background:
This research project is investigating canine fertility and environmental pollutants. We intend to examine canine fertility across a number of European countries in an effort to establish if there has been any change or abnormal development in canine reproductive organs. One of the measures to determine these changes are cellular abnormalities in testes. The tissue collected from your dog during this routine castration will be very useful to us in undertaking our investigation.

Please take a few moments to read the following and, if you are in agreement, sign below. I (the undersigned) agree to provide background data and tissue from my dog for the above research project being conducted on behalf of Guide Dogs for the Blind Association, the University of Nottingham and Nottingham Trent University.

I also understand that:
1. All information that I provide will be treated with respect and stored in a secure location.
2. All information obtained will be anonymous and confidential.
3. Once collected, all individual data will be aggregated and only the tissue collection reference number will be retained for identification.
4. Any personal information will only be used for the purposes of this study.
5. Only residual tissue from the castration will be collected and the dog will not have to undergo any additional procedures.

I agree to have data collected on the overall health of my dog as well as information related to the castration procedure. Please tick below as appropriate.

[ ] Yes I agree
[ ] No I do not agree

I agree that the residual tissue from the castration of my dog can be retained and used for this study. Please tick as appropriate.

[ ] Yes I agree
[ ] No I do not agree

Name of dog owner _____

Signature_____ Date _____

Thank you for your participation and if you require further information please contact Andrew Byers using this email address: andrew.byers@ntu.ac.uk.

Canine Fertility Data Sheet

Tissue Reference Number: DN001

General Information

Date of Birth of the dog? __ / __ / ____ (dd / mm / yyyy) **or Age if DOB unknown _____**

Breed? _____ Kennel Club Registration Number _____

Current Address (post code only) _____ Years at this address _____

Details of previous address(es) where the dog has lived (post code and number of years only) if known:

Postcode and/or location of dog's breeder if known _____

Does the dog live in the house? Yes ☐ No ☐

Dietary Information

My dog's diet consists of (e.g. 50% Canned commercial dog food & 50% Dry commercial dog food):

_____%	_____%	_____%	○
Canned commercial dog food	Dry commercial dog food	Human food	Other – please give details below

Dietary details:(feeding frequency, manufacturer, dietary supplements used)

Clinical History

Reason for castration? **Date of Castration? __ / __ / ____** (dd / mm / yyyy)

○	○
Routine	Other – please give details below

Details of reason for castration:

Does the dog have a history of reduced fertility or other reproductive problems?

○	○	○
No	Yes – please	Unknown

Details of fertility problems:

Does the dog have a history of non descended testes (cryptorchidism)?

○	○	○
No	Yes – please see below	Unknown

May we contact the veterinary practice who treated you dog for cryptorchidism, please provide practice details.

Does the dog have a familial history of fertility problems?

○	○	○
No	Yes – please see below	Unknown

Details of familial history of reproductive problems.

Has the dog any previous history of disease or ill health?

○	○	○
Yes – please give details	No	Unknown

Details of previous ill health:

Additional Health Information – *it would be extremely helpful to our research if you could provide some further details on your dog!*

Details of vaccination and anti -parasite programme:
Details of any medications given (trade names and dates):
Details of any past poisoning:
Are air fresheners or carpet treatments used routinely in the home? Type?
Details of Flea treatments/household flea treatments?
What type of bedding is used?
In the part of the home predominantly occupied by the dog, how would you describe the flooring?
Carpet ☐ , Tiles ☐, Laminate ☐, Wood ☐**,** Concrete ☐
Are there any smokers in the dogs household Yes ☐ No ☐ (if yes please see below)
Number of smokers in the household? _____ Average number of cigarettes smoked per person? _____
Details of pesticides or herbicides used in the garden?
Does your dog (tick as appropriate)?
drinks from puddles ---------- Yes ☐ No ☐ swims in canals/rivers/ponds ---------- Yes ☐ No ☐ swims in/uses treadmill at hydrotherapy centre - Yes ☐ No ☐

Protocols and data required from attending veterinarian

Weight? _____ kg Height at shoulder _____ cm

Body Score Condition _____ (see Figure 3 for guidance on BSC)

Preservation of tissue samples

Remove the epididymis from the testes and weigh both testes individually

Weight Left testis _____ g.

Weight Right testis _____ g.

Cut each testis in ½ and cut a 5 mm disc (approximately) from the face of each half (See Figure 2 below). (May be necessary to cut each 5mm disk in ½ to ensure it fits in tissue sample pot for fixation) In preparation for histological examination, chemical analysis and nucleic acid preservation, please carry out the following:

Histology examination:

Place each pair of disks, along with respective epididymis, in separate pots of Bouin's fixative. Label sample pot with date and time. Pots will be labeled as either 'left' or 'right' along with the Tissue Reference Number *which corresponds to the number on the tissue data sheet. Leave the sample in Bouin's for 6 hours then transfer to 70% ethanol. The sample can then be stored and shipped at room temperature.*

Chemical analysis:

Wrap 3 of the remaining 4 pieces of testis in aluminum foil, label and store below 0 C (standard freezer at – 20 C is fine). Theses samples can ultimately be shipped with freezer packs.

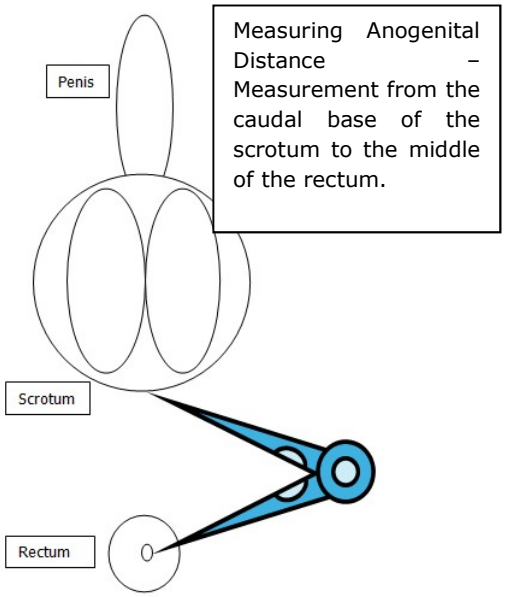
Nucleic acid preservation:

Cut or scrape a small fragment of peripheral testis (4x4x4 mm approximately) and transfer to the small plastic tube provided containing excess 'Qiagen Allprotect Reagent'. The samples can then be stored in a fridge (5 C) and shipped at room temperature. N.B. tissue sample must be completely submerged in the reagent.

Anogenital distance measurements

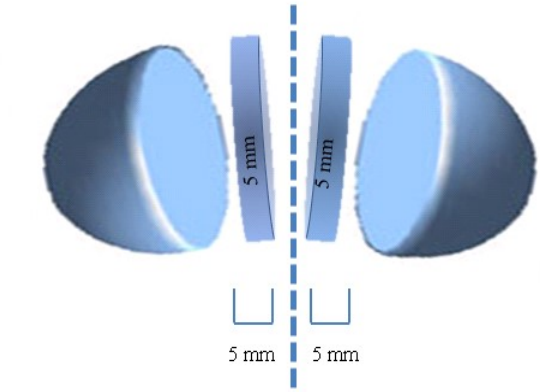
Measure anogenital distance as outlined in Figure 1 below. Take two separate measurements and record in table below. Please use the calipers provided.

Anogenital distance measure 1 _____ mm	Anogenital distance measure 2 _____ mm



Measuring Anogenital Distance – Measurement from the caudal base of the scrotum to the middle of the rectum.

Figure 1 Instruction for the measurement of anogenital distance



Cut each testis in ½ along the axil plane. Cut a 5mm disk off the face of each ½ testis prior to immersing disks in Bouin's fixative*

Figure 2 Instructions for dissection of the testes

depending on the testis size, some disk may have to be cut in ½ to fit in the tissue sample pot for fixation.

Figure 3 Instruction for the measurement of body condition

Body Score Conditioning



1 Very Thin

Ribs	Easily felt with no fat cover
Tail base	Bones are raised with no tissue between the skin and bone
Side view	Severe abdominal tuck
Overhead	Accentuated hourglass shape



2 Thin

Ribs	Easily felt with no fat cover
Tail base	Bones are raised with minimal tissue between the skin and bone
Side view	Abdominal tuck
Overhead	Marked hourglass shape



3 Ideal

Ribs	Easily felt with slight fat cover
Tail base	Smooth contour but bones can be felt under a thin layer of fat
Side view	Abdominal tuck
Overhead	Well proportioned lumbar waist



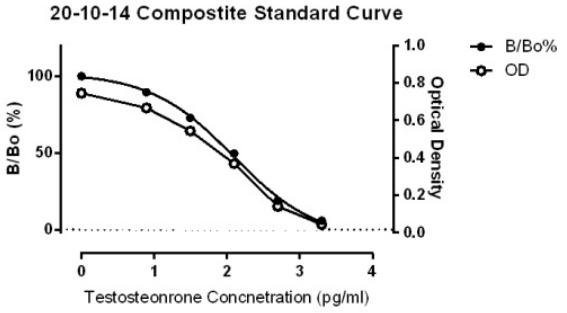
4 Overweight

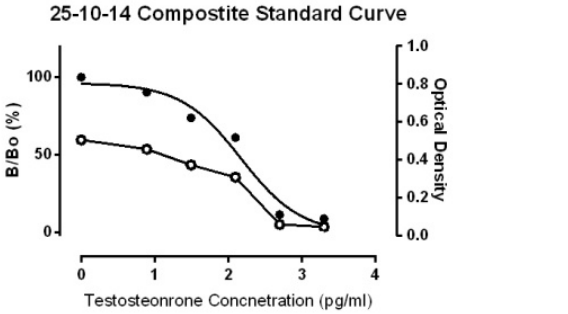
Ribs	Difficult to feel with moderate fat cover
Tail base	Some thickening but bones can be felt under a moderate layer of fat
Side view	No abdominal tuck or waist
Overhead	Back is slightly broadened

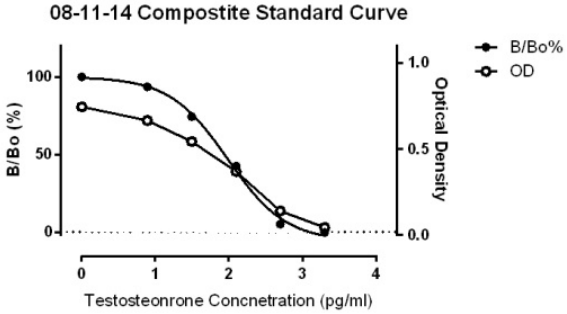


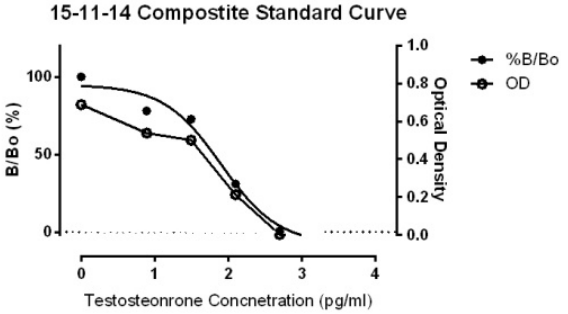
5 Obese

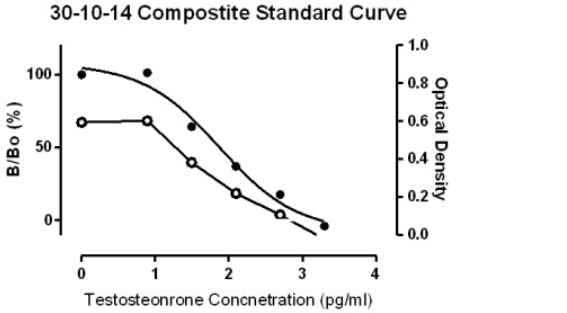
Ribs	Difficult to feel under thick fat cover
Tail base	Thickened and difficult to feel under a prominent layer of fat
Side view	Fat hangs from the abdomen and there is no waist
Overhead	Markedly broadened

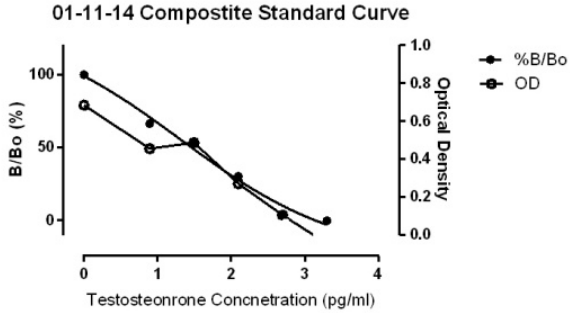

20-10-14 Compostite Standard Curve
B/Bo%
OD
B/Bo (%)
Optical Density
Testosteonrone Concnetration (pg/ml)

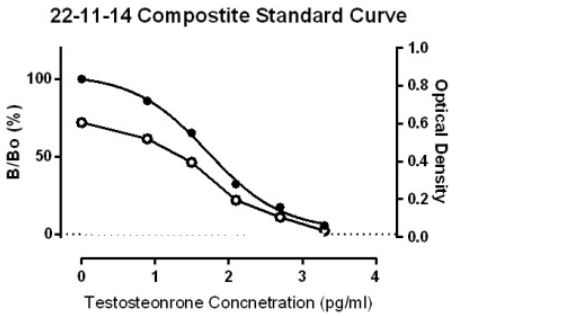

25-10-14 Compostite Standard Curve
B/Bo (%)
Optical Density
Testosteonrone Concnetration (pg/ml)

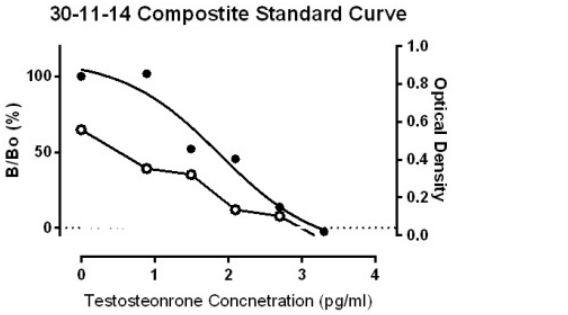

08-11-14 Compostite Standard Curve
B/Bo%
OD
B/Bo (%)
Optical Density
Testosteonrone Concnetration (pg/ml)


15-11-14 Compostite Standard Curve
%B/Bo
OD
B/Bo (%)
Optical Density
Testosteonrone Concnetration (pg/ml)


30-10-14 Compostite Standard Curve
B/Bo (%)
Optical Density
Testosteonrone Concnetration (pg/ml)


01-11-14 Compostite Standard Curve
%B/Bo
OD
B/Bo (%)
Optical Density
Testosteonrone Concnetration (pg/ml)


22-11-14 Compostite Standard Curve
B/Bo (%)
Optical Density
Testosteonrone Concnetration (pg/ml)


30-11-14 Compostite Standard Curve
B/Bo (%)
Optical Density
Testosteonrone Concnetration (pg/ml)

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Effects of Exposure to Environmental Chemicals During Pregnancy on the Development of the Male and Female Reproductive Axes

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Contents

There is a large body of literature describing effects of environmental chemicals (ECs), many of them anthropogenic with endocrine-disrupting properties, on development in rodent laboratory species, some of which lead to impaired reproduction and adverse health. This literature joins extensive human epidemiological data and opportunistic wildlife findings on health effects of ECs. In contrast, the effect of endocrine disruption on foetal development and reproductive performance in domestic species is less extensively documented. This applies both to domestic farm and to companion species even though the former is critical to food production and the latter share our homes and many aspects of the modern developed human lifestyle. In domestic species, the nature of chemicals exposure *in utero* and their consequences for animal health and production are poorly understood. A complication in our understanding is that the pace of development, ontogeny and efficiency of foetal and maternal hepatic and placental activity differs between domestic species. In many ways, this reflects the difficulties in understanding human exposure and consequences of that exposure for the foetus and subsequent adult from epidemiological and largely rodent-based data. It is important that domestic species are included in research into endocrine disruption because of their (i) wide variety of exposure to such chemicals, (ii) greater similarity of many developmental processes to the human, (iii) economic importance and (iv) close similarities to developed world human lifestyle in companion species.

Introduction

A wide range of environmentally ubiquitous chemicals (ECs) are classified as endocrine-disrupting compounds (EDCs). These comprise diverse groups of chemicals, primarily anthropogenic in origin, which are used in a wide range of commodities and processes (fire retardants, softening agents in plastics, pesticides, etc.) and are released as a result of fossil fuel combustion associated with transport and power generation. Other compounds, which might not be expected to have endocrine-disrupting actions, include nitrates (Edwards et al. 2006), which are commonly used as fertilizers, and some analgesic pharmaceuticals (Kristensen et al. 2011). All have one feature in common – the capacity to interfere with normal hormonal processes (Rhind 2009): the actions of these chemicals impinge on ecosystem sustainability (Rhind 2009), farm animal productivity (Rhind et al. 2010a) and human fertility and health (Toppari et al. 1996). The *in utero* and post-natal growth of an individual is reliant upon a highly regulated sequence of signalling processes to ensure appropriate organogenesis, and programming for adult health and function. Endocrine signalling is an important part of these processes and, in this review, we present some of the evidence for endocrine disruption of foetal reproductive development observed in domestic species.

Exposures to Environmental Chemicals

There are numerous reports of physiological disruption associated with 'exposure' to individual chemicals, as a result of accidental release (Guillette et al. 1994), and mixtures of chemicals put into the environment through waste recycling (Rhind et al. 2010a) but in many of these cases, the actual concentrations of chemicals in the environment are poorly defined or were not measured. Even in more controlled, laboratory-rodent studies involving the administration of individual chemicals, 'exposure' is generally defined in terms of the dose applied rather than the concentrations that actually reach the target tissues (Gray et al. 2000).

Clearly, measurement of concentrations of selected chemicals in relevant environmental matrices may provide some indication of animals exposure but many EDCs degrade in the environment as a result of exposure to ultraviolet light or microbial activity while inputs of volatile compounds through aerial deposition occur continuously (Teil et al. 2006; Wang et al. 2007) and, therefore, spot sampling may not provide a meaningful measure of exposure. A true understanding of the physiological effects of EDCs, however, requires knowledge of the EDC concentrations at the site of action (i.e. tissue exposure/concentrations) When assessing tissue exposure (Fig. 1), it is necessary to take account of not only the environmental levels but also the likely rates of uptake through each of the probable routes of exposure (e.g. ingestion, inhalation and dermal absorption); the efficiency of transfer from the environment into the animal may differ with exposure route, diet, dermal characteristics and individual chemical (Rhind 2008). After EDCs enter the body, the capacity

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of the animal to absorb and catabolize or excrete chemicals of different classes and of their capacities to cross the placenta, blood–brain barrier or other physiological barriers may determine target tissue burdens (Rhind 2008).

Measurement of EDC concentrations in target organs is technically difficult and costly. In view of the large numbers of chemicals known to have disrupting effects, measurement of almost any group of chemicals is likely to provide no more than an indication of the total EDC burden. Nevertheless, knowledge of tissue EDC burdens in both 'background-exposed' and 'highly exposed' animals, and of factors that affect these burdens, is essential for understanding of physiological responses to exposure. Such studies are logistically impracticable for most species of wildlife and are rarely conducted in laboratory-rodent studies. However, tissues of species such as domestic ruminants that contribute to the human diet are easily obtained and since knowledge of their EDC content is clearly relevant to human exposure as well as to the animals' physiology, some measurements of tissue levels in these species have been conducted.

In a series of studies, sheep were exposed to pastures fertilized with processed sewage sludge, which is known to contain large amounts of multiple EDCs (Stevens et al. 2003; Ghanem et al. 2007) or with inorganic fertilizer that contains only background concentrations. These studies have provided some insight into patterns of tissue accumulation. It has been shown that (i) the effects on tissue EDC burdens of increased exposure, through sludge-treated pastures, are subtle and often not statistically significant (Rhind 2008; Rhind et al. 2010a). (ii) There are species differences in metabolism and accumulation as indicated by the fact that relatively high tissue concentrations of diethylhexyl phthalate (DEHP) are present in sheep tissues irrespective of exposure to sludge (Rhind et al. 2005), signifying that, in this species unlike rodents, DEHP is not entirely degraded in the digestive system (White et al. 1980). (iii) Chemicals of several classes are present in foetal tissues, indicating that transfer across the placenta occurs (Rhind et al. 2009, 2010b). (iv) The efficiency of trans-placental transfer differs with chemical class, resulting in markedly different rates of accumulation (Rhind et al. 2010b). (v) Concentrations of many, but not all, EDCs are higher in maternal than foetal tissue (Rhind et al. 2009, 2010b). (vi) Some compounds (e.g. PCBs 101 and 118) are preferentially accumulated in foetal tissue, relative to maternal tissue (Rhind et al. 2009, 2010b). (vii) EDC concentrations depend on the phase of gestation at which the exposure occurs (Rhind et al. 2010c). It is also noteworthy that maternal-foetal and foetal–foetal relationships with respect to EDC burdens, within animals, are generally weak (Rhind et al. 2009, 2010b).

Collectively, these observations demonstrate that exposure to environmental concentrations of EDCs result in minimal increases in tissue concentrations of most compounds but patterns of accumulation are influenced by multiple factors. While such exposure frequently results in minimal changes in absolute tissue levels, exposure to multiple EDCs is associated with multiple disruptions in animal physiology.

Hypothalamo-Pituitary-Gonad Axis and Endocrine Disruption

Normal reproductive function is dependent on hypothalamic regulation of gonadal function via secretion of gonadotropin-releasing hormone (GnRH) and the pituitary gonadotrophins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which then acts on the ovaries or testes to stimulate gonadal maturation, gametogenesis and steroidogenesis. The hypothalamo-pituitary-gonad (HPG) axis is regulated by numerous excitatory and inhibitory hypothalamic inputs and highly sensitive to the organizational and activational effects of endogenous steroids during foetal and perinatal life (MacLusky and Naftolin 1981; Robinson et al. 2002). Extraneous influences on any tier of HPG axis during critical periods of development have the potential to permanently alter the 'programming' of normal reproductive development resulting in dysregulation of reproductive function and reduced fertility in later life. As such, the HPG axis is well placed as a target for the action of EDCs during critical periods of HPG axis development. Studies using individual chemicals have demonstrated the susceptibility of the HPG axis in domestic species (e.g. (Collet et al. 2010)) to EDCs, which are present in the environment (Table 1). The significance of studies examining effects of individual chemical exposures on the HPG axis is difficult to determine as domestic species, like humans, are not exposed to one chemical but rather a mixture of chemicals throughout different life stages that can have additive effects even at low (NOAEL) levels (Rajapakse et al. 2002). For instance, octylphenol exposure *in utero* suppresses foetal FSH secretion, testis size, Sertoli cell number (Sweeney et al. 2000) and puberty timing later in life (Wright et al. 2002). *In utero* bisphenol-A (BPA) and methoxychlor (MXC) exposure is associated with a reduction in hypothalamic GnRH expression (Mahoney and Padmanabhan 2010) and reduced basal LH concentrations and pulse frequencies (Cooke et al. 1996). Higher circulating BPA levels in women with polycystic ovarian syndrome (PCOS) compared to controls and a significant positive association between androgens and BPA point to a potential role of this EDC, via androgenic actions, in PCOS pathophysiology (Padmanabhan et al. 2010; Hogg et al. 2011).

Sheep exposed to the cocktail of chemicals contained in sewage-sludge-treated pastures have demonstrated that such exposure has negative effects on the HPG axis. Offspring from mothers exposed to sewage sludge chemicals were associated with perturbations at several levels of the HPG axis, including altered hypothalamic and pituitary expression of the neuropeptide kisspeptin and its receptor (Bellingham et al. 2009), which are vital for the central regulation of GnRH neurosecretory activity and timing of puberty (Messager 2005; Roa et al. 2008) in adulthood. The same foetal animals also exhibited perturbation of other neurotransmitter systems involved in regulation of GnRH (Bellingham et al. 2010). Sewage-sludge-exposed foetuses also had altered gonadal development; reduced number of Leydig and Sertoli cell numbers correlated with reduced testosterone production in males (Paul et al. 2005) and altered

Table 1. Summary of endocrine disruption in sheep

Chemical	Dose/route	Species	Associated	References
Bisphenol-A	> 38 ng/ml (i.v. infusion)	Sheep	↓ LH Pulse Frequency ↓ Basal LH Secretion	Collet et al. (2010)
	5 mg/kg/day (i.m.) (gestation d30–d60)	Sheep	↓GnRH expression[a] ↑Oestrogen Receptor 1 Expression (POA)[a] ↓Oestrogen Receptor 2 expression (POA)[a]	Mahoney and Padmanabhan (2010)
	3.5 mg/kg/day (i.m.) (from 4 to 11 weeks old)	Sheep	↓ LH secretion ↓ LH pulse frequency and amplitude	Evans et al. (2004)
Methoxyclor	5 mg/kg/day (i.m.) (gestation d30–d60)	Sheep	↓GnRH expression (adult offspring) ↑Oestrogen Receptor 1 Expression (POA) ↓Oestrogen Receptor 2 expression (POA)	Mahoney and Padmanabhan (2010)
Octylphenol	1000 μg/kg day (i.v. infusion) (gestation d70–birth)	Sheep	↓FSH[b] ↓Testis Weight[b] ↓ Sertoli cell number[b]	Sweeney et al. (2000)
Sewage Sludge Application to pasture	Unknown (*in utero* via maternal exposure)	Sheep	↓ Kisspeptin expression (hypothalamus)[b] ↓Kisspeptin/LH colocalization (pituitary)[b]	Bellingham et al. (2009)
		Sheep	↓GnRH expression (hypothalamus)[b] ↓Galanin receptor expression	Bellingham et al. (2010)
		Sheep	↓Ovarian development[b]	Fowler et al. (2008)
	Unknown (*in utero* and lactational exposure)	Sheep	↓ Germ cell/Sertoli Cell[a] ↑Histopatological observations[a]	Bellingham et al. (2011)

[a]Adult offspring.
[b]Foetal offspring (Evans et al. 2004).

ovarian development in females (Fowler et al. 2008, 2011). A subsequent study in adult rams, exposed *in utero* and via lactation, demonstrated an association between sewage sludge exposure and reduced testicular germ cell numbers (Bellingham et al. 2011), yet this was not associated with any effect on sperm counts in these animals. However, it should be noted that domestic ruminants store sperm and so may appear to have a high sperm count even when sperm production is reduced. Coincidently, a large proportion of genital abnormalities ranging from testicular hypoplasia to hypospadias were found in rams in abattoirs in the UK over a period of 4 years (Smith et al. 2011) and although the authors do not speculate about the involvement of EDC exposures as having a potential causative role, given that sewage sludge application to pasture is common practice throughout the UK and the similar findings of (Bellingham et al. 2011), it is not unreasonable to suggest that EDCs may play a role in the manifestation of such abnormalities.

Effects of Exposure to EDCs During Pregnancy on Gametes and Early Embryonic Development

The foundations for gametogenesis are established during foetal development and it is increasingly recognized that environmental insults during pre- and perinatal phases may have a subsequent impact on the quality of gametes. In fact, while the accompanying effects of adulthood exposure are in all probability reversible, exposure of the developing gonads leads to irreversible effects on germ cell quality. Furthermore, gamete quality is critical to generate a developmentally competent zygote. This means that perturbations induced by endocrine disruptors during germ cell formation can cause permanent alterations in reproductive performance visible only in adults. Finally, such changes in the germ line could be transmitted to subsequent generations, causing a transgenerational effect in the progeny. Almost all the *in vivo* studies on the effects of prenatal exposure to EDCs on gamete quality and early embryonic development in F1 animals have been conducted in laboratory rodents. However, there have been recent reports of *in vitro* studies correlating EDC exposure to gamete quality in farm animals (Campagna et al. 2001; Pocar et al. 2001, 2003; Alm et al. 2002; Wang et al. 2005), and these results are consistent with *in vivo* observations in rodent models.

In vivo studies with laboratory rodents have shown that direct exposure to certain classes of EDC during pre- and perinatal development induced deleterious effects in F1 animals of both sexes, leading to altered reproductive performance later in life. In females, *in utero* and lactational exposure to phthalates and polychlorinated biphenyls (PCBs) reduced oocyte maturation and developmental competence of mouse offspring (Pocar et al. 2011; Pocar et al. 2012a,b). Maternal exposures affect the earliest stages of oogenesis in the developing foetal ovary, and the resulting subtle meiotic defects increase the likelihood that embryos produced by the exposed female in adulthood (i.e. the grandchildren) will be chromosomally abnormal. BPA acts to down-regulate mitotic cell-cycle genes, raising the possibility that foetal BPA exposure may act to limit expansion of the primordial germ cell population (Lawson et al. 2011). Mouse oocytes exposed to BPA, *in vitro* or during foetal development, fail to properly align chromosomes at the spindle equator at MII and show synaptic defects such as end-to-end chromosome associations and incomplete synapsis (Susiarjo et al. 2007; Hunt et al. 2009). Furthermore, BPA-exposed oocytes are more prone to miscarriage than non-exposed oocytes (Hunt et al. 2003; Mlynarcikova et al. 2009).

In the human foetal ovary, BPA induces delay of meiotic progression of oocytes characterized by increments of the oocytes at leptotene, diminution of the percentage of oocytes that reached pachytene and decreased gamete viability. This aspect is accompanied with over-expression of *SPO11*, *H2AX*, *RPA* and *BLM*

in human foetal ovarian cultures. Since these genes are involved in the double-strand break generation, signalling and repair during meiosis and mitosis, their up-regulation could disrupt the cell cycle, cell survival and differentiation (Brieno-Enriquez et al. 2011, 2012). In domestic animals, a variety of *in vitro* studies have demonstrated that exposing oocytes to different classes of EDCs, including organochlorine pesticides, PCBs and p-tert-octylphenol (OP), during culture adversely affects maturation and subsequent developmental competence in cattle (Pocar et al. 2001, 2003; Wang et al. 2005) and in pigs (Campagna et al. 2001; Alm et al. 2002). These data nicely correlate with an *in vivo* study of OP effects in pigs where *in utero* exposure induced a reduction in litter size in F1 progeny consistent with altered oocyte maturation and embryonic development (Bogh et al. 2001).

In males, a significant decrease in sperm quality in terms of concentration, viability and motility has been observed in both mouse and rat after pre- and perinatal exposure to phthalates (Pocar et al. 2011; Sainath et al. 2011; Pocar et al. 2012a,b) and PCBs (Hsu et al. 2007; Pocar et al. 2012a,b). In addition, Veeramachaneni et al. (1995) observed that *in utero* exposure to p-tert-octylphenol or to a mixture of EDCs in rabbits induced acrosomal malformations in F1 offspring. To our knowledge, there are few data on the effects of developmental exposure to EDCs on semen quality in domestic animals. Nevertheless, a correlation between environmental exposure and the reproductive performance of farm animals has been suggested. (Veeramachaneni 2000) identified the same acrosomal malformations previously observed in rabbits, exposed *in utero* to a mixture of EDCs and in a group of sterile or subfertile stallions suggesting that environmental exposure to EDCs may form the basis of reduced fertility.

Taken together, these data strongly indicate that exposure to a variety of environmental pollutants during gonad development may induce permanent alterations in gametes of both sexes as adults. Although there is limited evidence of a EDC-driven decline in the fertility of domestic animals, the possibility that these effects may become more marked in future cannot be ruled out and so, therefore, the role of increasing environmental pollution on the reproductive health and breeding performance of farm animals cannot be ignored.

Endocrine Disruption in Companion Animals

In contrast to humans and a wide range of wildlife species, published evidence of impaired fertility in companion animals (dog, cat) is more limited. There is evidence that, over the last 40 years, the incidence of testicular tumours seen in dogs has increased in parallel with changes seen in humans (Grieco et al. 2008). Since environmental factors have been linked to the increased incidence of human testicular dysgenesis syndrome (TDS, Sharpe and Skakkebaek 2008), it is tempting to conclude that environmental factors probably account for the rise in incidence of testicular cancer in dogs. While this hypothesis requires further testing, histological characteristics of human TDS have recently been described, with the identification of seminiferous tubule abnormalities and carcinoma *in situ* (CIS) cells, in canine testes (Grieco et al. 2008). Since CIS cells are known precursors to human seminomas (Looijenga 2009) TDS is also likely to predispose the canine testis to cancer. Testicular dysgenesis syndrome includes a reduction in accepted indicators of fertility such as semen quality (Sharpe and Skakkebaek 2008). This raises the question of whether companion animal fertility is also on the decline since the dog and cat, through cohabitation with humans, are exposed to the same EDCs with potentially similar consequences for reproductive health.

A wide range of EDCs have been detected in both dog and cat tissue and serum (Table 2). In urban areas, canine lungs are contaminated with airborne polycyclic aromatic hydrocarbons (PAH), largely released from car engines, and dioxins associated with waste incineration (Takagi et al. 2004). The flame retardant PBDE is detectable in serum from both cats (Dye et al. 2007; Venier and Hites 2011). Interestingly, feline studies indicate that serum levels of PBDE may be associated with feline hyperthyroid disease (Dye et al. 2007), although a recent study did not show a similar relationship (Guo et al. 2012). This was attributed to possible metabolic differences in hyperthyroid cats, age and the measurement of PBDE congeners in serum rather than in the thyroid gland. Of note, however, is that PBDE concentrations in cats are reported to be comparable to those found in human infants, and this has been attributed to proximity to household materials treated with PBDE-based flame-retardants and higher levels of dust consumption (Frederiksen et al. 2009). In addition, polychlorinated biphenols have been detected in adipose tissue from cats and dogs (Storelli et al. 2009). Interestingly, higher levels in cats were attributed to the better ability of dogs to metabolize some of these contaminants. This could also be attributed to higher levels of dust consumption by cats because of grooming behaviour.

Although environmental contaminants can come from a variety of sources (e.g. dust, household material and occupational exposure), a major source of potential contamination is diet. Bisphenol-A has been detected in fifteen commercially available cat foods and 11 commercially available dog foods (Kang and Kondo 2002). Further research, using a narrower range of commercially available pet foods also found BPA present in some samples but at lower levels (Schecter et al. 2010). The differences in the level of BPA detected likely reflect different brands of food, processing differences or even analytical differences. Regardless, there is little doubt that BPA is present in canned pet foods and additional studies report the presence of other contaminants in pet food including PCBs, DDE and heavy metals (Mumma et al. 1986; Sakai et al. 1995; Lopez-Alonso et al. 2007). Moreover, possible implications of these detected levels of contaminants have raised concerns to the extent that owners are recommended to reduce their pets' consumption of canned food (Edinboro et al. 2004).

Diet has been established as a primary route for contamination of certain EDCs (e.g. BPA) in humans and particularly in children (Wilson et al. 2007). Diet has also been identified as a primary route of

Table 2. Range and levels of EDCs found in companion animal tissue and serum

Companion species (tissue)	EDC category	∑/congener	Levels (mean)	References
Canine (lung)	PCB	Congener		Takagi et al. (2004)
		BaA	0.19 ng/g	
		BkF	0.08 ng/g	
		BaP	0.1 ng/g	
	Dioxin	BghiP	0.1 ng/g	
		PCDD	6.0 pg/g	
		PCDF	1.12 pg/g	
		DL-PCB	70. 2 pg/g (wet weight)	
Feline (serum)	PBDE	∑ 14 congeners (9 congeners predominant)	10.5 ± 3.5 vs 12.7 ± 3.9 ng/ml[a] (non-HT vs HT)	Dye et al. (2007)
Feline	DDT	*p,p*-DDE	94.8 ng/g lipid wt	Storelli et al. (2009)
Feline	PCB	∑ 11 congeners	199.0 ng/g lipid wt	
Canine (adipose tissue)	PCB	∑ 11 congeners	41.6 ng/g lipid wt	
Canine (serum)	PBDE	∑ 6 congeners	1.76 ± 0.39 ng/g[a] (wet weight)	Venier and Hites (2011)
Canine (adipose tissue)	PCB	∑ 59 congeners	Range: 50 ng/g (range 60–153: control) vs 2995 ng/g lipid wt (range 936–6740)[b,c]	Kirkegaard et al. (2011)
Feline (serum)	PBDE	∑ 9 congeners	6709 ± 3319 vs 3461 ± 485 ng/g lipid wt (non-HT vs HT)	Guo et al. (2012)
	PCB	∑ 8 congeners	203 ± 44.6 ng/g vs 242 ± 31.8 ng/g lipid wt (non-HT vs HT)	

HT, hyperthyroid cats.
[a]Mean ± standard error.
[b]Effects of feeding minke whale blubber vs porcine fat (control).
[c]Congeners also detected in thyroid gland – data not tabulated.

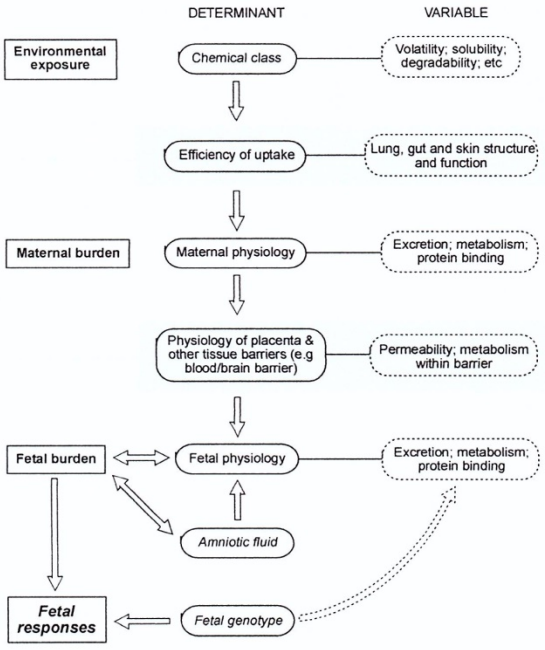


Fig. 1. Overview of factors that influence EDC uptake and rates of tissue accumulation in the dam and foetus

contamination in companion animals (Verreault et al. 2008, 2009). These researchers fed captive sled dogs a diet of porcine fat (control) or a diet of Minke whale blubber (treatment) and measured a range of EDCs in the adipose tissue (Kirkegaard et al. 2011). A clear correlation was demonstrated between high levels of organohalogen contaminants in the Minke Whale blubber and in adipose tissue from dogs on this diet, as well as their pre- and post-weaning pups (Verreault et al. 2009). Of particular note is that these findings equate with levels of environmental contaminants measured in Greenland people eating a traditional diet that includes marine mammals (Bonefeld-Jorgensen 2010).

The World Health Organization has put forward guidelines to evaluate safe exposure levels for certain EDCs using toxic equivalency factors (Van den Berg et al. 2006). The data used to create these recommendations are largely based on non-physiological experimental levels and the testing of single chemicals. Real-world exposure is, however, characterized by exposure to mixtures of chemicals at low environmental concentrations. As concentrations measured in companion animals are similar, the possibility remains that they may be able to perturb reproductive function in dogs and/or cats. However, if metabolic differences are responsible for the discrepancy in somatic burden of EDCs between companion species, this could suggest that the cat is a better sentinel for overall environmental contamination whilst the dog is a better indicator of bioaccumulation of EDCs in humans.

Evidence for Epigenetic Effects of Environmental Chemicals

As for *in vivo* experiments of prenatal exposure to EDC on gamete quality, almost all studies investigating putative epigenetic programming of reproductive development have been conducted in rats and mice, and this topic has been extensively reviewed elsewhere (Guerrero-Bosagna and Skinner 2009; Zama and Uzumcu 2010; Fowler et al. 2011). Consequently, reference to this subject will focus upon salient points of relevance to reproduction in domestic species that have arisen from these studies with rodents and which highlight the need for similar investigations in our farmed animals. Firstly, there is now compelling evidence from rodents to suggest that at least some of the actions of ECs on developmentally important genes may be mediated, at least in part, via epigenetic modifications to DNA methylation and various covalent modifications, including methylation, to associated proteins. Secondly, given the recent and imminent releases of draft pig and sheep genome sequences, respectively, and recent developments in 'next-generation' sequencing technologies, genome-wide analyses of epigenetic states in domestic species have now become feasible. Finally, evidence from rodent studies supports the concept of transgenerational germ-line transmission of epigenetic modifications, which may be sexually dimorphic (e.g. (Anway et al. 2005)). These observations should help to usefully direct similar studies in long-lived large animal species where the outcome of early-life epigenetic reprogramming of gene expression on adult physiological can also be expected to alter during the natural course of ageing and may be further modified by the prevailing endocrine milieu.

Conclusions

The current state of knowledge for the effects of EDCs on domestic animal development and reproduction is that weight of evidence supports concern that exposure to chemicals prenatally, and especially when combined with continuing post-natal exposures, is having adverse effects on animal function. However, as in humans and laboratory species, the links between phenotypic consequences and specific chemicals are difficult to make, and chemical cocktails are probably of much greater concern and species and sex differences complicate interpretation.

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Conflicts of interest

None of the authors have any conflicts of interest to declare.

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Endocrine disruptors and ovine reproductive development

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Summary

Declining fertility and perturbations in reproductive development in a variety of species have been linked to exposure to endocrine disrupting chemicals (EDCs), which are ubiquitous in the environment. Ruminants are largely exposed to such chemicals in sewage sludge fertiliser widely used in animal production systems. This has been investigated experimentally through the deliberate exposure of pregnant ewes to sewage sludge fertilised pastures or control pastures treated with inorganic fertiliser containing no detectable EDCs. Perturbations in the developing fetal hypothalamic-pituitary-gonadal axis have been observed in both male and female fetuses. A sub-population of rams exposed both pre- and post-natally exhibited adversely altered testis development. Periods of developmental sensitivity to EDCs have also been identified. An *in vitro* culture system for ovine fetal ovaries has shown that mixtures have a greater effect than individual chemicals. The dog provides a sentinel model of human dietary exposure to chemicals in ruminant-derived food products. Evidence of altered fertility and testicular cancer, along with EDC detection in dog testes and pet foods, support this concept. Established rodent models of EDC exposure provide a means to investigate mechanisms and transgenerational effects. Overall, monitoring and *in vitro* studies carried out in sentinel species and on human tissues, combined with *in vivo* mechanistic studies carried out in ruminants and rodents, provides a clear picture of the global impact of EDCs on reproductive development. In particular, the ovine model of sewage sludge exposure provides the only currently available "real life" model of exposure to a cocktail of EDCs.

Introduction

Since the publication of Silent Spring in 1962, perturbations in reproductive development in a variety of species have been increasingly associated with exposure to anthropogenic environmental chemicals (Carson 1962). The periods of fetal and early post-natal development are particularly sensitive to such exposures and this reflects the exquisite control of fetal

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organogenesis mediated by endocrine, metabolic and other key biological signalling processes. The term "endocrine disrupting chemicals (EDCs)" is frequently used to describe those chemicals which interfere with the endocrine system, however it is now clear that many of these chemicals perturb a wide range of other biological processes and physiological systems including gametogenesis, steroidogenesis, the neuroendocrine and immune systems as well as nutrient partitioning and metabolism. Since all of these processes are crucial for reproductive development, EDCs have the potential to impact on fertility at multiple levels.

Exposure to environmental chemicals

Chemicals described as EDCs are extremely heterogeneous and originate from a diverse range of sources including plastics, industrial lubricants and solvents, detergents, adhesives, pesticides, cosmetics, fertilisers and even analgesic pharmaceuticals. The main route of exposure to EDCs is through the diet (including by drinking), however less characterised modes of exposure also occur through the inhalation of industrial volatile organic chemicals, the production of which has markedly increased in recent years, and by absorption through the skin (Rhind et al. 2010). Ruminants are exposed to EDCs largely through modern day domestic animal production systems, reflecting the replacement of costly inorganic fertilisers with processed human sewage sludge (reviewed in Rhind et al. 2010). Sewage sludge contains a cocktail of thousands of environmental chemicals, thus constituting a major source of exposure that may be higher (i.e. accumulating over time) than found in the environment (Rhind et al. 2013). Exposure largely occurs through the ingestion of soil and to a lesser extent herbage, and this level of exposure has been described as relevant to "real life". Indeed a controlled sewage sludge experimental model has been characterised as a model for human exposure as described in detail below. Ruminants will also be exposed to pollutants through air inhalation although the level of such contamination has not been characterised.

This review is designed (1) to update current understanding on the impact of EDCs on ruminant reproductive development and production and (2) to discuss the relevance of data obtained from ruminant studies to other species, including humans. The relative advantages and disadvantages of alternative animal models will be reviewed and the ruminant work placed into perspective in relation to the global problem of environmental contamination with EDCs.

Ruminants and endocrine disruptors

An experimental ovine model of exposure to a mixture of EDCs was developed by exposing ewes to pastures to which sewage sludge had been applied. Although a variety of pollutants have been detected in sewage sludge, its application had minimal short-term effects on soil chemical concentrations when compared to control pastures fertilised with inorganic fertiliser (Rhind et al. 2002, Stevens et al. 2003). Similarly, liver concentrations in ewes grazing the pastures showed only slight changes compared to controls (Rhind et al. 2005, Rhind et al. 2009). Despite these minor effects of sewage sludge exposure on maternal and fetal liver concentrations, the exposure of pregnant ewes from conception to euthanasia at mid or late gestation, or the continued exposure of the offspring to weaning, markedly perturbs the development of all organs of both the male and female reproductive axes (see below).

Similarly, EDCs have been detected in bovine tissues at low concentrations and are derived from contaminated pastures (Petro et al. 2010). Given the low levels of EDCs in sheep purposely exposed to sewage sludge and showing clear effects on reproduction, chemical measurements

alone (frequently at only one end-point) do not preclude an environmental impact on ruminant fertility and reproduction. It is of note that polychlorinated biphenyls (PCBs) in milk are elevated in high-producing dairy cows compared to lower yield dairy cows (Petro et al. 2010). This reflects the negative energy balance and associated lipolysis that characterises high milk yield cows and is thus a potential source of exposure for the human consumer. Organochlorine pesticides and PCBs have been detected in bovine and ovine semen and human studies indicate that poor sperm quality may be associated with direct exposure to environmental contaminants (Rozati et al. 2002, Kamarianos et al. 2003b, Zhang et al. 2006). In examining bull fertility in a limited geographic region, a strong statistically significant correlation between the levels of pesticide used and the number of spermatozoa in semen was found (Snoj et al. 2013). Overall these data indicate that the multifactorial problem of declining fertility in the dairy cow is likely to include one or more environmental components.

Effects on the male

In the human male, exposure to EDCs has been associated with increasing incidences of testicular cancer, congenital abnormalities at birth (cryptorchidism, hypospadias) and a decline in sperm counts in adulthood. Since these problems cluster in specific geographical areas, they probably have a similar aetiology and are collectively referred to as testicular dysgenesis syndrome (TDS) (Skakkebaek et al. 2001). The rapid period of time over which this has occurred is indicative of an environmental, rather than principally genetic, cause and there is a vast literature based upon a variety of animal models. Although the decline in sperm counts has received the most publicity, it is also the most controversial. Historically, interest in declining human sperm quality stems from a meta-analysis of studies looking at sperm counts published in 1992 (Carlsen et al. 1992). Carlsen reported a decline in human sperm counts between 1938 and 1990 and this was further corroborated by the addition of 47 more studies and re-analysis in 2000 (Swan et al. 2000). A number of additional studies have been published, the most notable being that of Rolland et al. showing a decline in semen quality across France between 1989 and 2005 (Rolland et al. 2013). Nevertheless, these findings have been criticised on the basis of inconsistencies in andrology methods and changes in laboratory quality assurance during the period of analysis (Pacey 2013). More recent studies have cast further doubt on the precise nature of the changes in sperm counts, suggesting a modest reversal (Jorgensen et al. 2012). However, they nevertheless show that a worryingly large proportion of young men have below optimal sperm quality. In contrast, studies showing an increased incidence of human male congenital abnormalities at birth (especially in small for gestational age neonates) and testicular cancer are more robust and widely accepted (Jackson et al. 1986, Bergstrom et al. 1996). A large proportion of genital abnormalities have been reported in rams examined in UK abattoirs (Smith et al. 2012), including hypospadias, and these may reflect exposure to EDCs. There is also evidence of a decline in bull semen quality between 1970 and 1985, although post 1985, a number of sperm parameters improved while sperm morphology declined (Wahl & Reif 2009). The variability may reflect potential methodological inconsistencies as suggested for the human. An additional confounder in such studies is the culling of sub-fertile animals which may account for the lack of such a trend in an earlier study (Setchell 1997, Karoui et al. 2011).

The sewage sludge model of EDC exposure has been used to investigate the impact of a cocktail of environmental chemicals on testis development. The exposure of pregnant ewes to sewage sludge fertilised pastures from conception to day 110 of gestation reduced both the number of Sertoli cells and Leydig cells in the fetal testis and the hormones produced by

these cell types: inhibin A and testosterone respectively (Paul et al. 2005). The perturbation of Sertoli cell function has serious implications for adult male fertility. Sertoli cells are critical for germ cell development and, subsequently, sperm production. Consequently, perturbed Sertoli cell proliferation and/or differentiation will impact on spermatogenesis in adult life. Recently, the period of sewage sludge exposure was extended to include exposure via the mother from conception and the post-natal period until weaning (Bellingham et al. 2012). This ensured that all periods of Sertoli cell proliferation and development were included (Fig. 1) and 5 of 12 exposed rams were shown to exhibit significant testicular abnormalities manifest by fewer germ cells, calculated per testis or Sertoli cell, and increased numbers of tubules that only contained Sertoli cells. While these data support the concept that the germ cell is a key target for EDCs (Lagos-Cabre & Moreno 2012), it is uncertain why only a subset of animals appears to be susceptible to EDC exposure. Nevertheless, this finding is more similar to human than rodent studies and factors including differences in grazing or ingestion patterns or differences in maternal and/or fetal metabolism may be responsible. With respect to the latter, polymorphisms in metabolic genes have been reported in humans, e.g. *CSTP1*, *CSTM1* genes and these may modulate polycyclic aromatic hydrocarbon-DNA adducts (PAH-DNA) in human mononuclear white blood cells (Butkiewicz et al. 2000). Although sperm counts were unaffected in the 12 rams, repeated ejaculations were not tested and the finding probably reflects the capacity of the ruminant to store sperm.

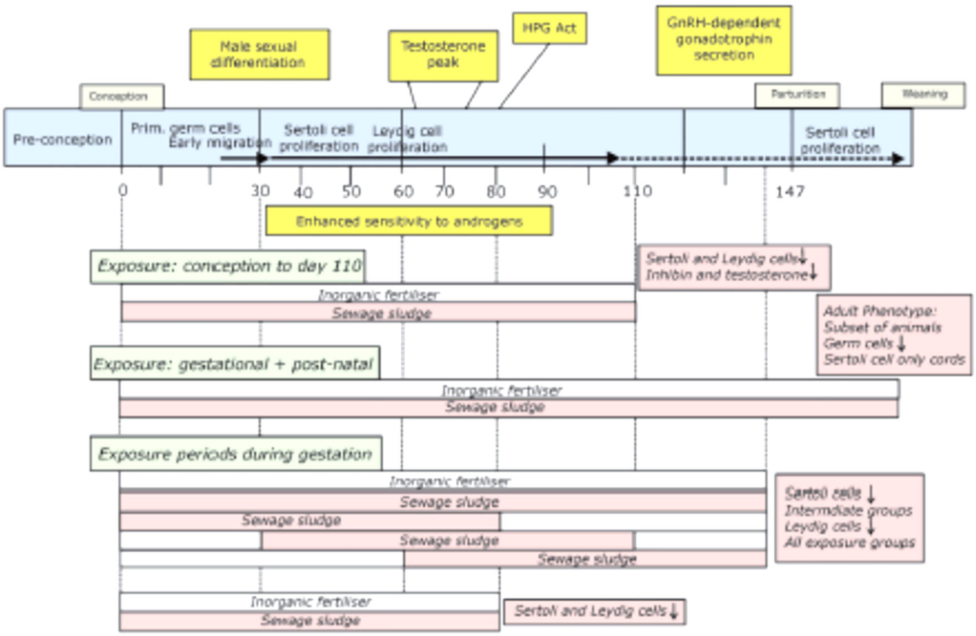


Fig. 1. The effects of EDCs contained in sewage sludge on the developing ovine fetal and post-natal testis. A developmental timeline is presented along with the experimental design and key observations. Pregnant ewes were exposed to sewage sludge fertilised pastures or to control pastures with inorganic fertiliser. Results are presented relating to (a) exposure of the ewes from conception to day 110 followed by euthanasia, (b) exposure throughout pregnancy followed by offspring exposure to weaning and (c) exposed for 80 day periods during early, mid or late pregnancy after which all ewes were euthanized at day 140. A separate subgroup was euthanized at day 80.

These findings raise the following questions: (a) which period(s) of gestation are more sensitive to exposure to EDCs and (b) do EDCs have different mechanistic effects at different stages of gestation. The key stages of fetal testis development are summarised in Fig. 1. To investigate this, we set up an experiment to investigate the impact of exposure of pregnant ewes to sewage sludge during 80-day windows in early, mid or late gestation periods (0-80, 30-110 and 60-140 days) and additional groups comprised ewes exposed continuously (0-140 days) and control non-exposed animals (0-140 days). Our preliminary findings indicate that numbers of Sertoli cells at day 140 of gestation were reduced in the 0-80, 30-110 and 60-140 exposed groups but not in animals continuously exposed from 0-140 days (Lea et al. 2010, R G Lea, B Loup, L Purdie, B Mandon-Pepin, M R Amezaga, S M Rhind, C Cotinot, P A Fowler & K D Sinclair 2014, unpublished observations). In contrast, a sub-group of animals exposed to sewage sludge for 80 days and immediately euthanised, did show reduced numbers of Sertoli cells relative to controls. It is possible therefore that between day 80 and day 140, the Sertoli cells underwent increased proliferation so that no difference was observed at the later time point. Leydig cell numbers showed a different pattern to Sertoli cells and were reduced in animals exposed throughout gestation and in the intermediate groups (Lea et al. 2010, R G Lea, B Loup, L Purdie, B Mandon-Pepin, M R Amezaga, S M Rhind, C Cotinot, P A Fowler & K D Sinclair 2014, unpublished observations).

In the sewage sludge model, two chemicals were present at greater concentrations in fetal compared to maternal livers: 2 polychlorinated biphenyl (PCB) congeners (PCB101, 118) and di(2-ethylhexyl)phthalate (DEHP) (Rhind et al. 2009). This observation provided the basis for a second ovine model whereby pregnant ewes were exposed from conception to late gestation (day 140) to environmental concentrations of DEHP, PCB (101,118) or a combination of the two. Our preliminary findings indicate that fetuses from ewes exposed specifically to each chemical independently or to a combination of the two, from conception to day 140, exhibit decreased numbers of Sertoli cells (Lea et al. 2011). This contrasts with the Sewage sludge exposure model where no differences in Sertoli cell numbers were observed at the same stage of gestation. Although the reason for this intriguing difference between the two ovine models is uncertain, it clearly reflects the increased complexity of exposure to a cocktail of EDCs in model 1 as compared to the more controlled exposure to two selected chemicals as described in model 2.

Effects on the fetal hypothalamus and pituitary gland

Exposure to sewage sludge is associated with perturbations of the fetal hypothalamus and pituitary gland. The regulation of reproduction, by both internal and external environmental factors, occurs at the level of the hypothalamus via gonadotrophin releasing hormone (GnRH) which in turn regulates gonadotrophin (LH and FSH) release from the pituitary gland. Sewage sludge exposure is associated with reduced expression of hypothalamic *GNRH1* mRNA and pituitary gland *GNRHR* mRNA (Bellingham et al. 2010), both of which might result in decreased drive within the hypothalamic-pituitary-gonadal axis. The GnRH neurosecretory system is not directly regulated by steroids and therefore not at direct risk of steroidogenic EDC effects. However, the above studies also documented reduced mRNA expression of the neuropeptide kisspeptin (*KISS1*) (Bellingham et al. 2009); a steroid-sensitive GnRH afferent and key regulator of the changes in GnRH neuronal activity required for pubertal transition and the generation of the preovulatory LH surge (d'Anglemont de Tassigny et al. 2007, Roa et al. 2011). The kisspeptin system is an important potential target for endocrine disruption, which could result in altered reproductive function (Tena-Sempere 2010, Losa et al. 2011). The effects of sewage sludge at the level of the hypothalamus is further supported by downstream effects in the pituitary

gland, manifest by decreased numbers of LHB immunopositive cells, a reduced number of cells co-expressing LHB and kisspeptin and reduced LHB/oestrogen receptor alpha (ESR1) co-expression in sewage sludge exposed fetuses relative to control animals (Bellingham et al. 2009). These changes could have negative effects on normal regulation of reproductive function. Further effects at the level of the hypothalamic-pituitary gland were seen relative to galanin, a neurotransmitter system involved in the relay of information about the internal environment to the GnRH neurosecretory system. Maternal sewage sludge exposure was associated with reduced fetal mRNA expression of galanin receptor isoforms in both the hypothalamus and pituitary gland (Bellingham et al. 2010). These studies demonstrate that continuous maternal grazing on sludge treated pastures before and during pregnancy is associated with significant alterations of fetal hypothalamic and pituitary regulators of GnRH function. Due to the sensitivity of the fetus to exogenous factors and developmental plasticity, such effects could have long term consequences for the reproductive neuroendocrine axis.

Effects on the female

A number of reports claim a link between exposure to endocrine disruptors and breast cancer, precocious puberty, premature menopause, PCOS, infertility and endometriosis in women (Crain et al. 2008, Bourguignon et al. 2013, Braun et al. 2013, Caserta et al. 2013). Rodent studies have shown that perinatal exposure to environmental chemicals induces precocious puberty, hastens reproductive senescence and induces other abnormalities of female reproductive development (Dickerson & Gore 2007, Gore et al. 2011). However, the data are contradictory and the evidence for a consistent negative effect of EDCs at sub-toxicological doses on fertility in women is controversial (Mascarenhas et al. 2012, Rostad et al. 2013). Part of the problem in humans is the variability and effects of a large number of confounding factors, including occupational vs environmental exposures, many of which cannot be accurately determined. In addition, in human fertility studies the contribution of the partner has to be included in a way that does not directly translate to animal studies (Buck Louis 2013). In ruminants, some EDCs have been reported in bovine follicular fluid however in other papers, EDCs were below the limit of detection (Kamarianos et al. 2003a, Petro et al. 2012).

In female mammals, including the human, the two key events of ovarian differentiation i.e. oogonia meiosis and follicle formation starts during *in utero* development and only concludes when the oocyte is fertilized (Sarraj & Drummond 2012) (Fig. 2, 3). Meiotic cell division is unique to germ cells, allowing them to produce haploid cells for sexual reproduction. During fetal life, the first meiotic prophase occurs, and then oocytes may remain arrested at dictyonema for many years in long-lived species (Spiller et al. 2012). In contrast to rodents, early folliculogenesis occurs *in utero* in ruminants and humans (Fig. 3) (Sathananthan et al. 2000, Sawyer et al. 2002, Baillet et al. 2008), beginning with recruitment of pre-granulosa cells to the oocyte to form the primordial follicle (Pepling 2012). The pool of primordial follicles constitute the ovarian reserve throughout the reproductive period in females (Forabosco & Sforza 2007) and some are stimulated to grow and develop into primary, secondary or antral follicles. Environmental compounds could, potentially, perturb the function of each of the cell types involved (oocytes and follicular cells). Moreover, EDC exposure during the fetal period can reduce the female's lifetime reserve of oocytes, which cannot be renewed, unlike males in which continuous spermatogenesis may quench transient EDC effects.

In the sewage sludge model, exposure of the female fetus *in utero* from conception to day 110 of gestation disrupts fetal ovarian development (Fowler et al. 2008). In a follow-up study, the timing of exposure was investigated: ewes were exposed pre-mating, post-conception

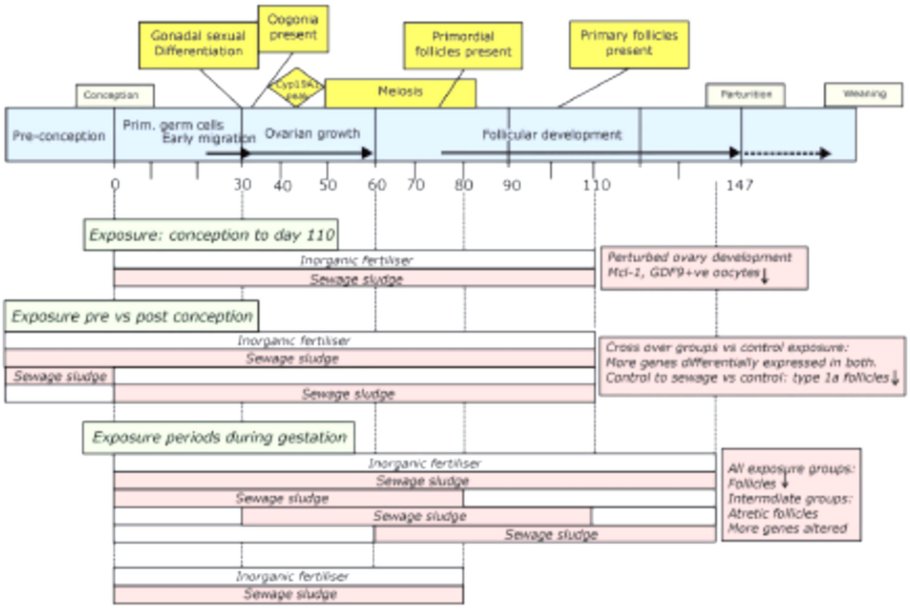


Fig. 2. The effects of EDCs contained in sewage sludge on the developing ovine fetal and post-natal ovary. A developmental timeline is presented along with the experimental design and key observations. Pregnant ewes were exposed to sewage sludge fertilised pastures or to control pastures with inorganic fertiliser. Results are presented relating to (a) exposure of the ewes from conception to day 110 followed by euthanasia, (b) exposure prior to conception or post-conception, with a cross over to control or sewage sludge exposure through to day 110, (c) exposed for 80 day periods during early, mid or late pregnancy after which all ewes were euthanized at day 140. A separate subgroup was euthanized at day 80.

or throughout both periods (Fig. 2). Ewes switched from control to sewage sludge at mating exhibited fewer follicles than those exposed throughout both periods (Bellingham et al. 2013). In addition, both cross exposure groups exhibited a greater number of gene expression changes compared to ewes continuously exposed to sewage sludge or control pastures. Thyroid glands from these animals were also most affected in these groups (Hombach-Klonisch et al. 2013). Consequently, a change in fetal ovarian environment, even to a reduced exposure to EDCs, appears to be more detrimental than a consistent period of exposure to normal or elevated levels of EDCs. Chronic exposure probably induces an increased detoxification in both mother and fetus highlighting the resilience capacity of the reproductive system.

Further recent studies have investigated which periods of gestation are the more sensitive to chemical exposure, using the experimental design as described above for males. Our preliminary findings indicate that follicle numbers are reduced in all exposure groups, with higher proportions of atretic follicles in the mid and late gestation groups. Moreover, transcriptomics revealed greater numbers of differentially expressed genes in the mid and late gestation windows. Thus similar to the pre- to post-conception switch over study, a change in environment appears to be more detrimental than continuous exposure. However, the preliminary data also suggest that primordial follicle formation, arrest and development may be particularly susceptible to exposure to EDCs

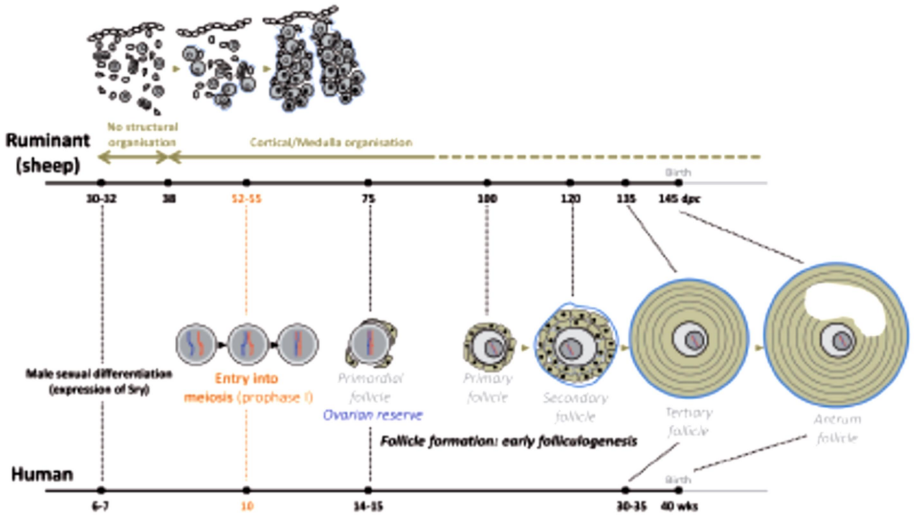


Fig. 3. Timeline of the major cellular events in mammalian ovary morphogenesis. During embryogenesis, the bipotential gonad is formed at the end of the first month of pregnancy in humans and sheep. The switch between male and female occurs a few days later. In the XX gonad, where *SRY* is absent, events in testis morphogenesis do not happen. Specification of the granulosa cell lineage defines the first step of ovary organogenesis (35-38dpc in sheep, 8 weeks in humans). This step is under the control of genes expressed specifically in the female such as *FOXL2* and *RSPO1*. Female germ cells within the germ cell nest begin to enter meiosis at 52-55dpc in sheep (9-10 weeks of gestation in humans) and arrest in the diplotene stage of meiosis I beginning at 75dpc (14-15 weeks in humans). The germ cell nests break down soon after to form primordial follicles. Follicle activation compartmentalizes the somatic cell environment, accompanied by the recruitment of theca cells to the primary follicle (100-110dpc in sheep, 20 weeks in humans).

contained within sewage sludge (Loup et al. 2011, R G Lea, M R Amezaga, B Loup, B Mandon-Pépin, M Fraser, C Kyle, C Kerr, M Osprey, Z Zhang, C Allen, L Purdie, K D Sinclair, C Cotinot, S M Rhind & P A Fowler 2014, unpublished observations). In a separate study, ovine female foetuses, maternally exposed to relatively low concentrations of bisphenol A (BPA), showed altered expression of ovarian steroidogenic enzymes and micro RNAs relevant to gonadal development and function. In support of the sewage sludge model data, gene expression investigated at days 65 and 90 revealed clear gestation day differences (Veiga-Lopez et al. 2013). Although we have not yet characterised a female adult phenotype in the sewage sludge model, pre-pubertal exposure to BPA or the organochlorine pesticide, methoxychlor (MXC), has been shown to alter post-pubertal reproductive function. This was manifest by a reduced LH surge amplitude in response to BPA and a delayed onset of the LH surge in response to MXC (Savabieasfahani et al. 2006). Interestingly, recent mechanistic studies indicate that this likely reflects altered follicular development and steroidogenesis in the ovary (Abi Salloum et al. 2013).

In vitro models parallel *in vivo* studies

Biologically relevant bioassays are needed for dose-response tests of EDC effects on steroidogenesis and gametogenesis and to understand the mechanisms of action by which EDCs

act on their targets to disrupt the normal processes of cellular differentiation. This has been demonstrated in the male where biologically relevant concentrations of the pesticide dieldrin have been shown to reduce testosterone secretion by human fetal testis explants (Fowler *et al.* 2007). However, because disturbances in the endocrine system can affect multiple systems, understanding the effects of EDCs is best accomplished by combining *in vitro* with *in vivo* approaches. Mouse follicle culture models are useful in studying folliculogenesis and oogenesis, (Cortvrindt & Smitz 2002) and in analysing the endocrine disruption of steroid biosynthesis (Myllymaki *et al.* 2005, Lenie & Smitz 2009). One mouse follicle culture model allows the individual and synchronous growth of a large number of follicles from early preantral to preovulatory stages of folliculogenesis (Cortvrindt & Smitz 2002). BPA exposed oocytes present unbalanced chromosome sets, decreased conceptions and are more prone to miscarriage than non-exposed oocytes *in vitro* (Can *et al.* 2005, Eichenlaub-Ritter *et al.* 2008, Lenie & Smitz 2009). Furthermore, effects of several PCB mixtures have been tested on *in vitro* maturation and fertilization of mouse oocytes (Kholkute *et al.* 1994, Kholkute & Dukelow 1997). The addition of PCB mixtures at concentrations ranging from 0.01 to 10 µg/ml, affected the fertilisability of the oocytes. The exposure of bovine oocytes to a mixture of PCBs showed a dose-dependent increase in the percentage of oocytes unable to complete the maturation process to metaphase II. Moreover, the adverse effects of exposure to PCBs extended to fertilization and further embryonic development even after the removal of PCBs from the culture medium (Pocar *et al.* 2001). Exposure of bovine cumulus-oocyte-complexes (COC) to coplanar PCBs affected maturation and a significant increase in apoptosis and *BAX* mRNA expression occurred at doses as low as 100.6 pg/ml (Pocar *et al.* 2005).

The development of an *in vitro* culture system mimicking cellular interactions and supporting the growth and differentiation of fetal ovaries is a crucial step for testing and understanding EDC effects. Indeed, *in vitro* culture systems allow kinetic and dose-dependent studies of toxicological agents in human fetal organs. Organ culture is an approach requiring a small quantity of biological material and may be an alternative to animal experiments. Several groups have developed human fetal ovarian *in vitro* cultures to test the effects of EDC exposures (Angenard *et al.* 2011, Brieno-Enriquez *et al.* 2011, 2012, Poulain *et al.* 2012). Human fetal BPA-exposed oocytes showed a delay in meiotic progression and oocytes exposed to BPA show an up-regulation of genes involved in double strand break generation, signalling and repair (Brieno-Enriquez *et al.* 2011, 2012).

The development and validation of a ruminant *in vitro* organ culture system allows individual chemical (BPA, DEHP and PCB) and mixture effects on early ovarian development to be tested (C Cotinot, personal communication). For this purpose, we optimised the culture conditions for two fetal sheep developmental stages (50-70 days and 90- 120 days post coitum (dpc) (Fig. 4). Day 50-70pc corresponds to prophase I of meiotic division of oocytes (11-22 weeks of development of human fetal ovaries, Fig. 3) while 90-120dpc corresponds to ovarian follicle formation (early folliculogenesis, 18-28 weeks in human, Fig. 3). Ovarian folliculogenesis is complex and is regulated by various endocrine, paracrine, and autocrine factors. For these reasons, we developed a long-term organ culture system in which three ovarian fragments per fetal ovary were placed on an insert floating at the surface of culture medium and maintained for 20 days (Fig. 4). This enabled the study of the onset and completion of prophase I meiosis (50-70 dpc), the transition of primordial to primary follicles (90-110dpc) and the transition of primary to secondary or antral follicles (110-130dpc). Mixtures of chemicals, at all environmental concentrations tested (10^{-4}M and 10^{-8}M), induced more changes in ovarian transcriptomes than chemical molecules tested alone (Mandon-Pépin *et al.* 2013). However, there were no significant differences in morphology or follicle numbers between exposed and non-exposed cultures.

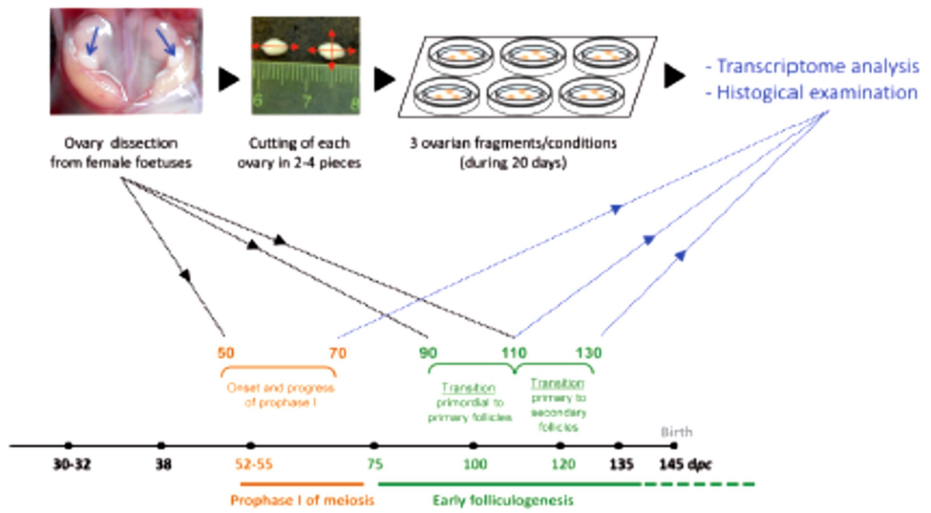


Fig. 4. Ruminant *in vitro* ovary culture system. The ovaries were removed aseptically from the fetuses at different developmental stages. Ovary explants were cut into pieces of a similar size, specifically two (50dpc), or four (90-130dpc) according to the stage. Three ovarian fragments were used for each condition. The filters bearing the explants were floated on 0.5 ml (early stages) or 1.2 ml (later stages) culture medium in tissue culture dishes and incubated at 37°C, in a humidified atmosphere containing 95% air/5% CO_2. The ovaries were cultured for 20 days with the medium being replaced once per day. Cultured ovaries were fixed in Bouin's fluid, embedded in paraffin and cut into 7-μm-thick sections for histological examination. Ovaries were also frozen at -80°C for molecular analyses.

The impact of BPA, DEHP and PCB on the fetal ovarian transcriptome is greater following *in vivo* exposure than following *in vitro* exposure. These differences may reflect i) chemical biotransformation in intact animals that does not occur in organ culture (metabolites were not tested *in vitro*); ii) other chemicals present *in vivo* acting in synergy with tested chemicals or their metabolites; iii) indirect effects *in vivo* through adverse effects on the hypothalamic-pituitary axis or other organs. These findings suggest that *in vitro* studies must be used for the understanding of the action mechanisms of one molecule rather than the characterization of gonad phenotype after exposure to a complex mixture.

Sentinels of EDC exposure: from ruminants to companion animals

In recent years, increased public awareness of toxic environmental chemicals has led to changes in manufacturing practices for food packaging, baby bottles and other products. It follows therefore that heightened awareness of EDCs in agriculture will focus further attention on domestic animal production systems and the potential consequences of dietary exposure to the consumer. A Swedish study examining levels of EDCs in food products identified fish as the primary source of dietary EDCs with dairy and meat coming second and third respectively (Tornkvist et *al*. 2011). While dairy products contain higher concentrations of polybrominated diphenyl ethers (PBDEs), they contribute less than meat overall due to consumption levels (Voorspoels et *al*. 2007). In Belgium, a range of EDCs have been detected in bovine tissues and in Scotland, a similar range has been detected in ovine meat albeit at variable levels depending

on the regional source (Petro et al. 2010, Rhind et al. 2011). Therefore the presence of EDCs in ruminant derived food products and contamination of agricultural land with toxic substances contained in processed human sewage sludge fertiliser, pesticides and herbicides emphasizes the importance and relevance of a ruminant sentinel model.

From the first reference in the academic literature a century ago of the canary being used to detect dangerous levels of carbon monoxide (Burrell & Seibert 1914), through the 'dancing' cats of the Minamata Bay region of Japan showing symptoms of methyl mercury poisoning (Rabinowitz et al. 2009), to bees and their honey being used as a biomonitor of pesticide use (Panseri et al. 2014) and brominated flame retardants in the environment (Mohr et al. 2014), the idea of a sentinel model is not new. In identifying an appropriate sentinel or biomonitor (term often used interchangeably with sentinel) for a target species, interspecies variability due to longevity, trophic level and diet, metabolism and habitat must be considered. Examples of sentinels are summarised in Table 1.

Table 1. Literature matrix showing strength of sentinel models with the human as the target species.[a]

Sentinel Model	Research Focus	Animal Category	Criteria Match	Reference
Feline	Low level chronic exposure to PBDE	Companion	Trophic level, Habitat	(Dye et al. 2007)
Canine	Metal concentration in tissue.	Companion	Trophic level, Diet, Metabolism, Habitat	(Lopez-Alonso et al. 2007)
Canine	PCBs	Companion	Trophic level, Diet, Metabolism, Habitat	(Schilling et al. 1988)
Canine/ Feline	TCDD	Companion	Trophic level, Diet,[b] Metabolism, Habitat[b]	(Schilling & Stehr-Green 1987)
Canine	Lead	Companion	Trophic level, Diet, Metabolism, Habitat	(Enriquez et al. 2009)
Ovine	Tissue levels of many EDCs, reproductive development and adult phenotype	Ruminant	Habitat: exposure to EDCs in sewage sludge fertilizer	(Rhind et al. 2009)
Bovine	Sperm quality	Ruminant	Habitat: as Ovine	(Wahl & Reif 2009)
Bovine	Tissue and fluid levels of PCBs, OCPs, PBDEs	Ruminant	Habitat: as Ovine	(Petro et al. 2010)
Primate	Germ cell differentiation/ testicular germ cell tumour	Wildlife	Longevity, Trophic level, Diet, Metabolism	(Mitchell et al. 2008)
Mink	Mercury, PCB	Wildlife	Trophic level, Diet	(Basu et al. 2007)
Polar Bear	OHCs	Wildlife	Longevity, Trophic level, Diet, Metabolism	(Sonne et al. 2006)
Marine Mammals	Anthropogenic toxins	Wildlife	Longevity, Species dependent	(Bossart 2011)
Domestic Animals	EDCs	General	Species dependent	(Majdič 2010)
Companion Animals	Public health/environmental contaminants	Companion	Species dependent	(Schmidt 2009)
Mammals	Toxic environmental contaminants	General	Species dependent	(O'Brien et al. 1993)
Animals	Environmental Chemicals	General	Species dependent	(van der Schalie et al. 1999)

[a]Criteria considered in model validity are longevity, trophic level, diet, metabolism and habitat. [b]Canine only. EDC: endocrine disrupting chemical, PBDE: polybrominated diphenyl ether, PCB: polychlorinated biphenyl, TCDD: tetrachlorobenzodioxin, OCP: organochlorine pesticides, OHC: organohalogen pollutants

Companion animals have been used as sentinel models for human exposure to EDCs. Established in Japan, the feline model is a useful sentinel for methyl mercury (Takeuchi *et al.* 1977) and PBDE in humans (Dye *et al.* 2007, Guo *et al.* 2012). Similarly, the dog has been proposed as a sentinel of human exposure to EDCs and we have recently identified a decline in canine semen quality which parallels that in humans (Byers *et al.* 2012) while an increased incidence of testicular tumours in dogs is evidence for canine TDS (Grieco *et al.* 2008). We have also identified specific EDCs in adult dog testes and the same chemicals in a range of commercially available dog foods is indicative of a nutritional link (Byers *et al.* 2012). Since the dog occupies the same environment as the human and is a carnivore, it may be an excellent sentinel of human exposure to EDCs particularly, but not exclusively, via the diet. Since a majority of the meat consumed by humans and companion animals is derived from ruminants, studies of the dog as a sentinel for human EDC exposure provides key information on the potential impact of ruminant meat-derived EDCs on the consumer. Interestingly, the accumulation of EDCs in Greenland people through the consumption of a high fat marine mammal based diet has been modelled by feeding Minke whale blubber fed to sled dogs (Verreault *et al.* 2008, Bonefeld-Jorgensen 2010). EDCs were not only traced through to adipose tissue in these animals but also to the offspring at pre- and post-weaning stages (Kirkegaard *et al.* 2011).

The use of sentinel models, especially for chronic exposure to ambient levels of environmental EDCs, provides insight not gained from experimental models. The canine sentinel model, based on the consideration of longevity, trophic level and diet, metabolism and habitat, is most useful in understanding EDCs and human health. The dog may be a useful biomonitor of dietary exposure for other target species such as polar bears (Verreault *et al.* 2008), which proves the versatility of these sentinel models. In the case of the ruminant, the bull has already contributed as a sentinel model to the weight of the argument suggesting a temporal decrease in semen quality.

Rodent models of EDC exposure: transgenerational and epigenetic effects

Many published studies on the effects of EDCs use rodent models, and often focus on single chemicals applied at relatively high concentrations *in vivo*. However, this by no means applies to all rodent studies. Indeed, a rat model of *in utero* exposure to di(n-butyl) phthalate at human relevant levels exhibits many of the features characterising human TDS (Fisher *et al.* 2003). Moreover a window of sensitivity (masculinization programming window) has been identified during which environmental chemicals are effective in perturbing testis development and inducing TDS like symptoms in male offspring (Welsh *et al.* 2008). This therefore provides a model whereby mechanisms can be established and tested in other sentinel and experimental model species. A key advantage of the rodent model is the investigation of mutigenerational or transgenerational effects of EDC exposure *in utero* and/or during lactation. Pocar *et al.* recently reported that the exposure of mice during pregnancy and lactation to a mix of PCB congeners (101 and 118) at concentrations comparable to human exposures impaired testis development, sperm counts, ovarian development and oocyte quality in F1 offspring (Pocar *et al.* 2012). Furthermore, reduced sperm viability and altered seminiferous tubule development up to the F_3 generation were observed.

There is compelling evidence from rodent studies that some of the effects of environmental chemicals, such as diethylstilbestrol (DES) (Alworth *et al.* 2002) and the isoflavonoid

phytoestrogen genistein (Tang et al. 2008), are mediated via epigenetic changes to DNA methylation. Anway and colleagues (Anway et al. 2005) went further to demonstrate male-germline mediated epigenetic transgenerational effects of the anti-androgenic compound vinclozolin (an agricultural fungicide) and the oestrogenic compound methoxchlor on gonadal sex determination, which persisted to the F_3 generation in the rat. However, because the F_0 gestating female is exposed to environmental stimuli, both the F_1 embryo and F_2 generation germ line are also directly exposed (Jirtle & Skinner 2007). Consequently, for epigenetic modifications to chromatin to be considered a plausible mechanism for transgenerational inheritance of phenotypic change, effects need to persist to at least the F_3 generation. However to date there is no direct link between exposure to environmental chemicals and epigenetic dysregulation of gene expression that relate to aberrant gonadal development and adverse fertility phenotypes in farm animal species. This is primarily because these mechanisms have not been investigated in this context in these species.

Conclusion: a global perspective on future studies

The evolutionary void between human and ruminant is even wider than between human and rodent. However, in many ways the patterns of reproductive and developmental events are much more similar between ruminant and human while key developmental events, such as primordial follicle formation, do not occur *in utero* but, instead, occur post-natally in the rodent. While in mechanistic terms there is much in common between ruminants, humans and rodents in terms of the processes, the events occurring post-natally in the rodent will, inevitably, be exposed to different chemicals (e.g. via lactation) and environmental influences than the human or ruminant e.g. direct exposure to the environment instead of *in utero* via the mother. In terms of digestion and metabolism there are, of course, some marked differences and similarities between ruminants, humans and rodents, such as hepatic recirculation (Mazur et al. 2010), which can be associated with considerable differences in internal chemical exposures despite a similar external dose. Notwithstanding the role of the rumen in exacerbating human-ruminant differences in digestion, internal dosage is still likely to be more similar between humans and ruminants. In one respect almost all species conduct their pregnancies at relatively low oestrogen levels compared with humans (human gestational steroid reviewed in Kuijper et al. 2013). In addition, the activity of the human fetal liver as a detoxification and steroidogenic organ (O'Shaughnessy et al. 2013) is markedly different from the rodent. The take-home message is that endocrine disruption is best studied in a number of laboratory and sentinel species in order to properly account for inter-species differences in exposure, internal dosage, metabolism and phenotypic response (Fig. 5). The sheep, especially housed outdoors with biosolids exposure is therefore an excellent model to better understand the reproductive consequences of endocrine disruption.

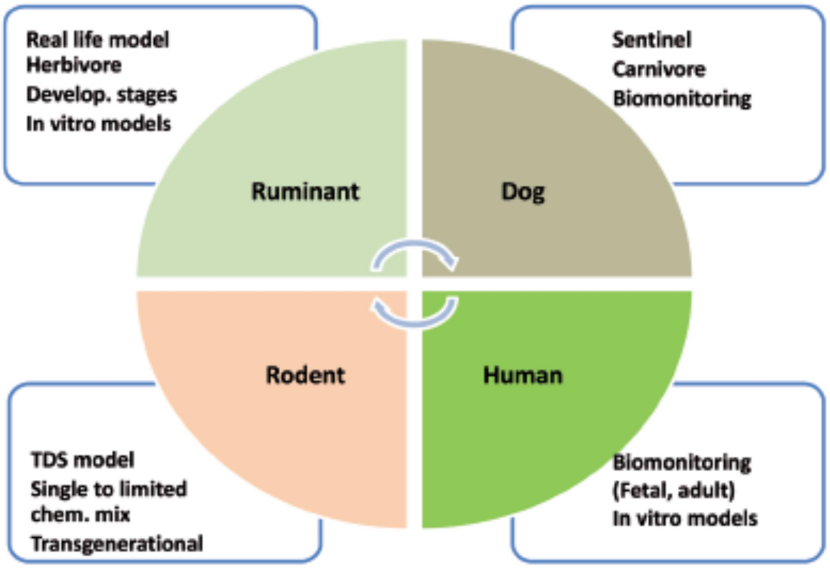


Fig. 5. Roles of experimental animal models and sentinel species in determining the reproductive impact of endocrine disrupting chemicals. **Ruminant:** Sheep exposed to a cocktail of chemicals contained in sewage sludge constitute a real life ruminant (herbivore) model of human exposure. The long period of gestation and well characterised stages of fetal development facilitate the elucidation of EDC effects at specific developmental stages. An established *in vitro* ovary culture system enables individual chemical and mixture effects to be determined. **Rodent:** An established rodent model of testicular dysgenesis syndrome (TDS) provides information on mechanism. The short gestation period has been exploited to investigate transgenerational effects. **Dog:** The dog is a recognised (carnivore) sentinel for human exposure to EDCs and gonad chemical analysis provides a means of biomonitoring. **Human:** Analysis of chemical profiles in adult and fetal samples/tissues provides a means of direct biomonitoring and gonad explant culture systems enable the direct effects of EDCs on human fetal tissues to be determined.

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Environmental chemicals impact dog semen quality in vitro and may be associated with a temporal decline in sperm motility and increased cryptorchidism

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Adverse temporal trends in human semen quality and cryptorchidism in infants have been associated with exposure to environmental chemicals (ECs) during development. Here we report that a population of breeding dogs exhibit a 26 year (1988–2014) decline in sperm quality and a concurrent increased incidence of cryptorchidism in male offspring (1995–2014). A decline in the number of males born relative to the number of females was also observed. ECs, including diethylhexyl phthalate (DEHP) and polychlorinated bisphenol 153 (PCB153), were detected in adult dog testes and commercial dog foods at concentrations reported to perturb reproductive function in other species. Testicular concentrations of DEHP and PCB153 perturbed sperm viability, motility and DNA integrity *in vitro* but did not affect LH stimulated testosterone secretion from adult testis explants. The direct effects of chemicals on sperm may therefore contribute to the decline in canine semen quality that parallels that reported in the human.

A significant decline in human semen quality over the last 70 years has been widely reported[1,2]. The first meta-analysis reporting this phenomenon included 61 studies selected over a 50 year period (1938–1991)[1] and such a trend was confirmed by re-analysis with an additional 47 studies included[2]. More recently, a decline in semen concentration and morphology has been reported across the whole of France over a 17 year period[3]. Despite these reports, differences in laboratory methodologies and standards, sometimes included in the same meta-analyses, question the reliability of some of these studies[4,5]. Nevertheless, reports of declining sperm counts linked with epidemiological data on increased incidences of testicular cancer and genital tract abnormalities, is indicative of an adverse environmental effect on male reproduction[6–8]. Since these reproductive problems, termed by some as "testicular dysgenesis syndrome" (TDS), cluster in geographical areas[9] they are thought to have a common aetiology and have been associated with endocrine perturbations in early life[10]. Exposure of developing males to environmental chemicals, particularly those with endocrine disrupting activity, is thought to be the initiator[11]. Although periods of development particularly sensitive to exposure encompass both pre-natal and pre-pubertal periods, environmental chemicals also perturb adult testis function e.g. gene expression and meiosis[12]. Although geographic divergences in exposure levels to oestrogenic or anti-androgenic compounds may explain the regional and national variations in reproductive health observed[13–15], the data demonstrating such a relationship remains contentious[4,16].

As 'man's best friend' and closest animal companion, the dog shares the same environment, exhibits the same range of diseases, many with the same frequency, and responds in a similar way to therapeutic treatments. There

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is evidence that over the last 40 years, the incidence rate of canine testicular cancer has increased in parallel with changes seen in humans[17,18]. In addition, histological signs which characterise human TDS have recently been described in the dog[19]. These include seminiferous tubule abnormalities and testicular germ cell neoplasia *in situ* (GCNIS) cells which are known precursors of seminomas in the human[20]. Since human TDS includes a reduction in sperm count[21] and an increased incidence of cryptorchidism, we hypothesised that the dog may exhibit similar manifestations of TDS and that this may be associated with exposure to endocrine disrupting chemicals. We therefore (1) monitored canine semen quality over a period of 26 years using standard and consistent techniques and (2) interrogated an extensive database of electronic health records for evidence of a temporal change in the incidence of cryptorchidism reported in male puppies. To explore a possible environmental aetiology, concentrations of chemical pollutants were measured in canine testes and semen collected from the same geographical area in which the temporal study had been carried out. The effects of chemicals, at testicular concentrations, were tested on endocrine and sperm function *in vitro*.

Results

Temporal changes in canine sperm motility, morphology and output. Sperm was collected from a population of stud dogs from a controlled breeding programme from 1988 to 2014. The coefficient of inbreeding for this population was low, with the annual mean coefficient for the 3 main breeds being: Labrador retriever = 0.05 (min 0, max 0.33); Golden retriever = 0.05 (min 0, max 0.34); German Shepherd Dog = 0.03 (min 0, max 0.28). The number of dogs within the programme each year varied between 42 and 97 throughout the period of study. Some dogs were examined during several consecutive years but the mean age of the population did not change each year because older dogs were retired and younger dogs were recruited to the breeding programme. Replacement dogs were selected based upon temperamental and health criteria but not fecundity.

A decrease in the percentage of sperm with normal forward progressive movement (motility) was observed between years 1988–1998 (2.5% per year) with years 1992–1998 being significantly lower than 1988 ($P < 0.05$: Fig. 1a). After removal of dogs with the poorest semen quality between 1999 and 2001, there was a further decrease in motility (1.2% per year) with years 2007 to 2014 being significantly lower than 2002 ($P < 0.05$) (Fig. 1a). Between 1988 and 1994, the mean percentage of live normal morphology sperm declined (8.1% per year) with years 1992 to 1994 being significantly lower than 1988 ($P < 0.01$) (Fig. 1b). However, between 1994 and 1998, the percentage normal sperm increased and plateaued but still remained significantly lower than in 1988. After removal of dogs with the poorest semen quality between 1999 and 2001, there was no further significant decline with time (Fig. 1b). An increase in the total sperm output (TSO) was observed between years 1988–1998 with years 1992–1994 being significantly greater than 1988 (Fig. 1c). After removal of dogs with the poorest semen quality between 1999 and 2001, TSO increased with years 2004 and 2005 being significantly greater than 2002. This was followed by a decrease in TSO from 2005 to 2006 after which TSO remained unchanged through to 2014. During this period, years 2006–2009 and years 2011 to 2014 were significantly lower than 2002 (Fig. 1c). For all three parameters (percentage normal motility, percentage normal sperm and total sperm output), there were significant differences between breed and a non-linear decline with age of the dog.

Internal quality control procedures were applied to evaluate intra-technician variation as well as variation between the three technical staff who carried out the sperm analyses. The mean annual intra-class coefficient correlations for intra-technician variation were: Motility = 0.78 (95% confidence interval 0.34–0.94), Morphology = 0.62 (95% confidence interval, 0.05–0.88), Total sperm output = 0.85 (95% confidence interval 0.54–0.96). Three technical staff were involved in the semen analysis with change-over years of 1998 and 2008. Figure 1 shows a very slight decline in motility and morphology in 2008, however this does not appear to impact on the temporal trends for each of the 2 parameters. Indeed, the intra-class coefficient correlations for inter-technician variation were: Motility = 0.74 (95% confidence interval 0.45–0.88), Morphology = 0.38 (95% confidence interval, 0.13–0.78), Total sperm output = 0.74 (95% confidence interval 0.38–0.93).

Temporal changes in the incidence of cryptorchidism and male/female ratio. The incidence rate of cryptorchidism in puppies from 1995 to 2014 was assessed from records maintained on the National Breeding Centre database (average number of male dogs per year = 651.25 ± 14.79). Figure 2a demonstrates a significant increase in cryptorchidism from 1995–2014 ($R^2 = 0.33$, $P = 0.008$, $n = 20$). Over the same period of time, there was a decrease in the proportion of total males born per litter (Fig. 2b: $R^2 = 0.20$, $P = 0.04$, $n = 20$, male:female ratio = number of males/total pup number per litter). However the decline was lost when stillbirths and post-natal mortalities were excluded. Further analysis of fetal and neonatal deaths revealed both a decline in male mortality (Fig. 2c: $R^2 = 0.32$, $P = 0.008$, $n = 20$) and an increase in female mortality (Fig. 2d: $R^2 = 0.31$, $P = 0.009$, $n = 20$).

Establishing concentrations of environmental chemicals in canine adult testis and semen. Canine testes were subjected to EC analysis to establish (1) presence in the tissue (2) if concentrations are compatible with those associated with infertility in the human and (3) to determine canine testicular concentrations for *in vitro* functional or viability studies with testis and sperm. Seven polychlorinated bisphenol (PCB) congeners, 5 polybrominated diphenyl ether (PBDE) congeners and diethylhexyl phthalate (DEHP) were detected in testis (Fig. 3a–c).

Sperm rich fractions (fraction 2) of 14 canine ejaculates were collected and two pools generated of 5 samples and one of 4 samples. This provided enough cellular material for chemical analysis of PCB and PBDE congeners (but not DEHP). All 3 pools had detectable levels of 7 PCB congeners including PCB 153 (13.05 ± 3.18) as well as PBDE 28 and PBDE 47. Two of the pools also had detectable levels of PBDE 99 and PBDE 100 (Supplementary Figure S1a,b).

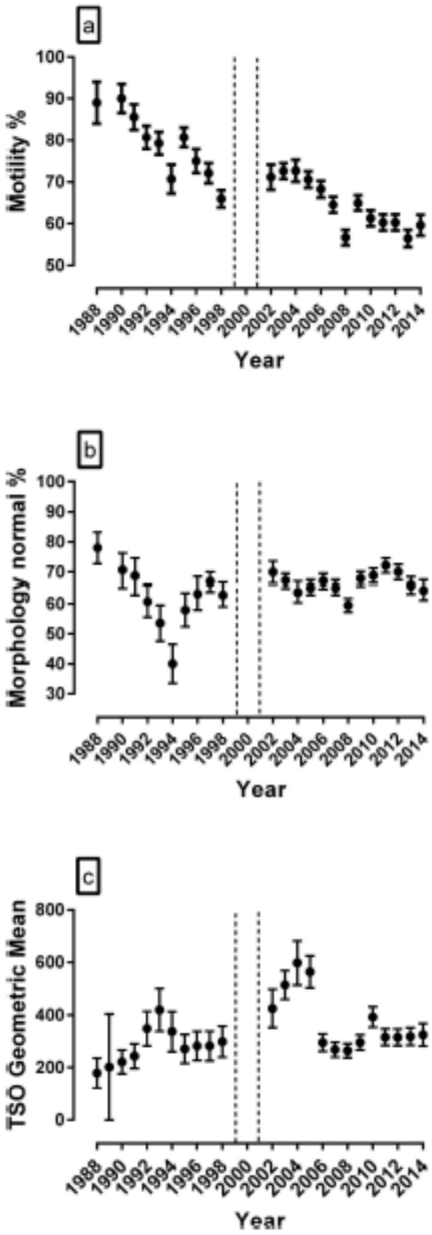


Figure 1. Temporal changes in semen quality in a population of stud dogs. Graphical summaries of the final statistical model parameters are shown for (a) Percentage Normal Motility, (b) Percentage Live Normal Sperm and (c) Total Sperm Output. In 1999 to 2001, dogs with poor semen quality were removed from the programme. Values of each parameter are given for years 1988–1998 and years 2002–2014 (n = 232 dogs: 42 to 97 in any one year). Statistical analysis: conventional multilevel models (MLwin version 2.22) used to evaluate change in semen quality over time accounting for repeated semen measurements. Error bars represent ± the standard error of each estimated mean point value from the model.

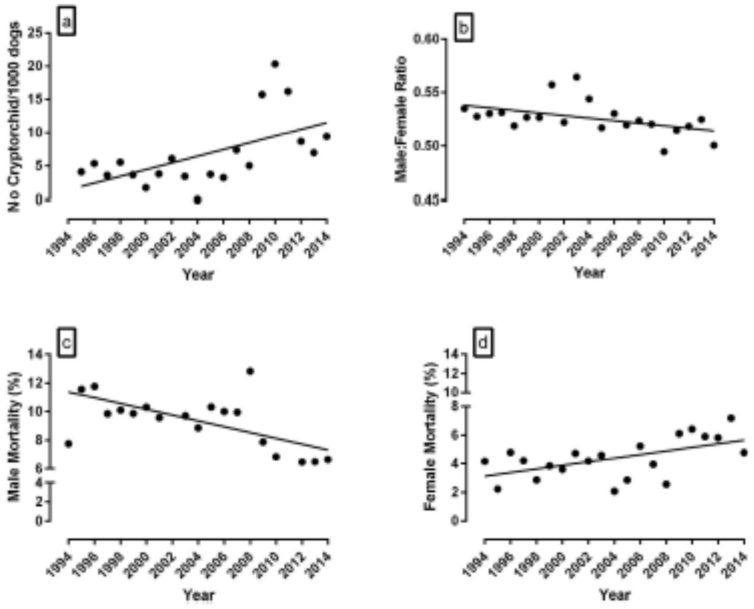


Figure 2. Temporal changes in the incidence of cryptorchidism, male:female ratio and mortality (stillbirth + early postnatal deaths) in puppies generated at the National Breeding Centre from 1994/5 to 2014 (**a**) Incidence rate of cryptorchidism ($R^2 = 0.33$, $P = 0.008$, $n = 20$). (**b**) Male:female ratio (No.Males/No.offspring per litter) of all pups born (including stillbirths and early postnatal deaths) ($R^2 = 0.20$, $P = 0.04$, $n = 20$). (**c**) Male mortality ($R^2 = 0.32$, $P = 0.008$, $n = 20$). (**d**) Female mortality ($R^2 = 0.31$, $P = 0.009$, $n = 20$). Statistical significance was assessed by linear regression (GraphPad Prism). Mean number of male dogs born per year (alive + still born) = 651.25 ± 14.79.

Since PCB153 and DEHP were detected in a majority of testis samples (Fig. 3a–c) and since PCB153 was also detectable in pooled ejaculate (Supplementary Figure S1a), these chemicals were selected at testis levels for *in vitro* studies using canine testis explants and sperm cultures. Mean testis levels for PCB153 and DEHP were calculated as 0.063 ± 0.01 ng/g (n = 34) and 0.182 ± 0.044 ug/g respectively (n = 25).

Establishing concentrations of environmental chemicals in commercial dog foods. To investigate a possible nutritional source of ECs, a range of commercially available dog foods were subjected to the same array of analyses as the testis and ejaculate samples. Consistent with testis and ejaculate measurements, seven PCB congeners, 5 PBDE congeners and DEHP were detected in dry (Fig. 3d–f) and wet (Fig. 3g–i) dog food respectively. Amongst the dry dog food samples, one sample designed for puppies (1 to 24 months of age) had higher concentrations of 4 PCB congeners (101, 118, 138, 153) and PBDE 47 relative to the other samples tested. One popular wet dog food had higher concentrations of PBDE 47 and all 7 PCB congeners relative to the other food brands tested. DEHP and some PCB and PBDE congeners predominant in some of the dry and wet foods were also predominant in a majority of testis samples. This included PCB153 and DEHP selected for *in vitro* studies.

PCB153 and DEHP induce contrasting effects on parameters of sperm movement measured by Computer Assisted Sperm Analysis (CASA). By CASA analysis (Hobsons sperm tracker), PCB153 accentuated percent linearity ($P < 0.05$), percent motility ($P < 0.05$) and straight line velocity ($P = 0.05$) (Fig. 4a,b,d). In contrast, DEHP reduced each measure of sperm movement reaching significance in percent linearity ($P < 0.05$), percent straightness ($P < 0.05$) and straight line velocity ($P < 0.05$) (Fig. 4e,g,h) but not in percent motility (Fig. 4f).

PCB153 and DEHP perturb sperm vitality and DNA fragmentation *in vitro* but do not affect testosterone secretion from testis explants. Canine sperm, collected immediately after ejaculation, was incubated with PCB153 or DEHP at 2x, 10x and 100x testicular concentrations. Sperm vitality was measured at 0 and 4 hours and DNA fragmentation at 0, 2 and 4 hours. Sperm vitality, measured by the hypo-osmotic swelling test, was significantly reduced by PCB153 ($P < 0.05$) (Fig. 5a) and DEHP ($P < 0.01$) (Fig. 5b). Sperm DNA fragmentation was

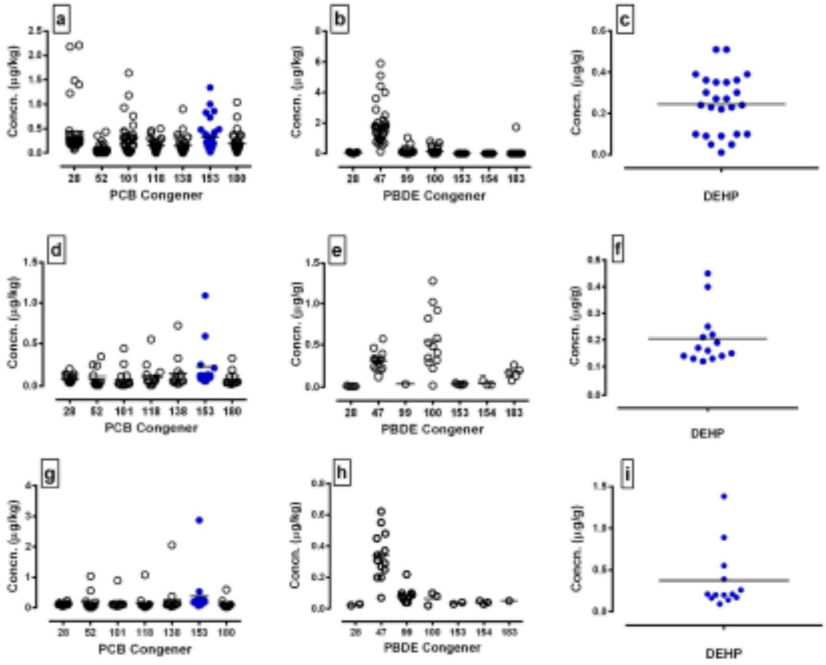


Figure 3. Concentrations of environmental chemicals in adult dog testes and commercially available dog food. (**a**,**d**,**g**) PCB congeners, (**b**,**e**,**h**) PBDE congeners and (**c**,**f**,**i**) DEHP were measured in (**a**–**c**) testes, (**d**–**f**) dry dog food and (**g**–**i**) canned dog food. Each circle represents a single testis or food sample. Blue circles show PCB 153 and DEHP that were detected above base levels in testes and food and selected for *in vitro* studies. Line indicates mean value.

increased following chemical exposure (overall treatment effect: $P < 0.001$, PCB153 and DEHP considered independently: $P = 0.05$) (Fig. 5c). There was no evidence of a difference with concentration or an effect of time.

Adult canine testes were maintained *in vitro* for 24 hours and normal endocrine function of the explants was demonstrated by a dose-dependent increase in testosterone in response to the addition of LH ($P < 0.01$). The addition of PCB153 and DEHP at 10x and 100x mean testis concentrations, to the explant cultures exhibited no inhibitory effects on LH-induced testosterone secretion (Fig. 5d).

Discussion

The findings from the current study are significant for the following reasons. They document, for the first time, a decline in the semen quality of a population of stud dogs over a period of 26 years. Although in some years semen quality parameters temporarily increased as noted in other studies[22], there was a repeated decline in motility even after removal of animals with the poorest semen quality. Contemporaneous with the decline in semen quality, offspring from the same population of dogs showed an increased incidence of cryptorchidism from 1995 (first available records) to 2014, a decline in the proportion of male pups at birth, and a temporal increase in female mortality. The decline in number of males relative to the number of females was lost when stillborn pups/early post-natal deaths were excluded. Significantly, we report that the endocrine disrupting chemicals PCB153 and DEHP are detectable in canine testes collected from dogs from the same location as the temporal study. Furthermore, the same chemicals were detected in a range of commercially available dog foods. Since testicular concentrations of these chemicals directly perturbed sperm motility and viability, this may be a mechanism by which environmental chemicals directly affect male fertility.

In the current study, it was not our intension to demonstrate temporal changes in semen or testicular chemical content and to correlate with changes in semen quality. Indeed, such screening is confounded by the need to pool individual semen samples, the lack of historical samples and access to the technology at the start of the study. Rather, we have focussed on temporal changes in semen quality and have demonstrated that ECs are able to influence motility and other parameters of semen quality. Similarly, correlating the decline in semen quality in individual stud dogs with cryptorchidism in their offspring would be difficult since an individual stud dog may have a breeding career of 8 years and may produce several hundred male offspring. Thus although the relationship between semen quality, cryptorchidism and ECs is not demonstrated through a longitudinal or cross-sectional

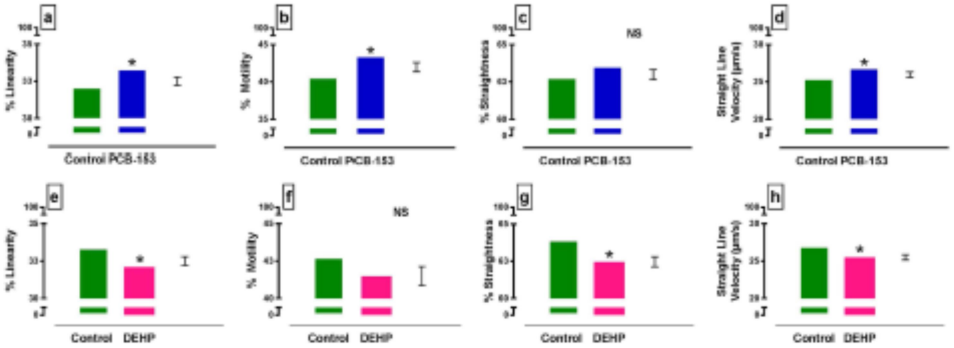


Figure 4. Computer Assisted Semen Analysis (CASA) of canine sperm exposed to testicular concentrations of PCB153 and DEHP. (**a**–**d**) Effects of PCB153 and (**e**–**h**) DEHP are shown for (**a**,**e**) percent sperm linearity, (**b**,**f**) percent sperm motility, (**c**,**g**) percent straightness and (**d**,**h**) straight line velocity. Overall chemical effects are shown (n = 7 dogs). Note: chemicals were tested at 2x, 10x, 100x mean testis concentration [mean testis levels for PCB153 0.063 ± 0.01 ng/g (n = 34) & DEHP: 0.182 ± 0.044 ug/g (n = 25)]. There was no interaction with concentration or time. Predicted values from general ANOVA model are presented. *P < 0.05. NS = not significant. Error bar = SED.

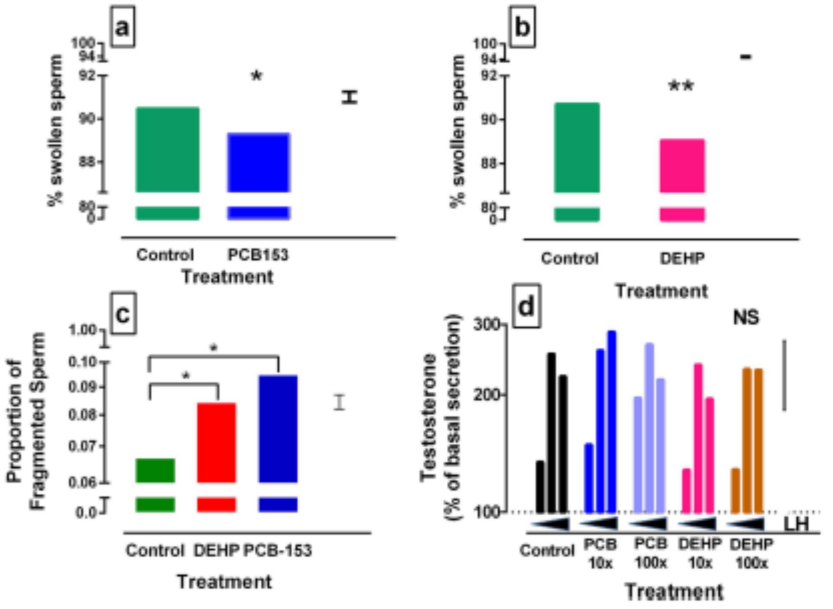


Figure 5. Effects of testicular concentrations of PCB 153 and DEHP on sperm vitality, DNA fragmentation and testosterone secretion from testis explants. (**a**,**b**) Sperm vitality was measured by the hypo-osmotic swelling test following incubation with PCB 153 (**a**) and DEHP (**b**) [n = 7 dogs]. (**c**) Sperm DNA fragmentation was measured by sperm chromatin dispersion assay (n = 10 dogs). (**d**) LH stimulated testosterone secretion by canine testis explants was measured in the presence of each chemical independently. Dotted line (100%) = explant + media only (no LH, no chemical). Chemicals were tested at 2x, 10x and 100x (**a**–**c**) or 10x and 100x (**d**) the concentration detected in testis [mean testis levels for PCB153 0.063 ± 0.01 ng/g (n = 34) & DEHP: 0.182 ± 0.044 ug/g (n = 25)]. (**a**–**d**): Predicted values from general ANOVA model are presented. *P < 0.05, **P < 0.01. NS = not significant. Error bar = SED.

study design, we postulate that the rapidity of the temporal changes observed are indicative of an environmental aetiology and that our *in vitro* data support this contention.

The original meta-analysis reporting a decline in human semen quality spanned a period of 53 years (1938–1991)[1]. Although this time frame is extensive, the study outcomes have recently been criticised on the basis of changes in laboratory methods, training of laboratory personnel and improved quality assurance over the years[4]. Since additional studies have either supported or refuted the original claims, declining human semen quality remains controversial. In contrast, data reported here on canine sperm quality have been generated using sperm analysis methods that have remained consistent throughout the 26 years of the study with uniform input from three experienced technical staff and one of the senior authors (GE). Indeed, although there was some variability between technical staff, this was in line with, or better than, other studies[23]. Given that the current study is devoid of the confounding factors inherent in human meta-analyses, we present a unique dataset showing a reliable temporal decline in semen quality which, in this case, was observed in a controlled population of stud dogs. This raises the tantalising prospect that the decline in canine semen quality has an environmental cause. Indeed it is unlikely that the effect is heritable since in this population of dogs heritability measures were low for all parameters except for the heritability of high sperm motility and low total sperm output[24].

The decline in percent normal morphology sperm from 1988 to 1994 followed by an increase and apparent subsequent plateau is difficult to interpret. Although a temporal decline in human sperm morphology has been reported and attributed to environmental factors[3], the degree to which this accounts for the temporal trends in canine sperm morphology is uncertain. Furthermore, the increase in total sperm output contrasts with the decline in motility and morphology and with human studies and meta-analyses showing a decline in sperm counts. One factor that may have influenced the increased sperm output from 2002 to 2005 is that between 1999 and 2001 dogs with poor semen quality were removed. Since this included dogs with less than 150 million sperm, their removal may have constituted a bias towards increased sperm output. Indeed a similar temporal increase in sperm output has been reported in bull semen between 1985 and 1995, and a comparable selection bias was suggested[22].

In the human, cryptorchidism affects 2 to 9% of new born infants[25] and published data generally supports a temporal increased incidence and clear geographic differences in the prevalence[25,26]. As a key manifestation of "testicular dysgenesis syndrome" in the human, data presented here illustrates a similar increased incidence of cryptorchidism in a population of stud dogs comprising 5 breeds. While inbreeding in dogs has been linked to cryptorchidism, it is generally accepted that the pathology is underpinned by an interaction between genetic, epigenetic and environmental factors[27]. It is however difficult to tease out the relative contributions of these drivers. Whilst we were unable to undertake chemical analysis on the cryptorchid testes, it is an interesting observation that all of these cryptorchid pups were produced from the stud dogs donating the semen and described in Fig. 1. Clearly, study of the effect of exposure of the pregnant dam on male fetuses are warranted. In a study of 1,339 litters of 4 different canine breeds, litters with 1 or 2 cryptorchids were reported to exhibit an imbalanced sex ratio in favour of males[28]. In a separate canine study in which cryptorchidism carriers were intentionally selected for further breeding, a similar increased male to female ratio (in favour of males) was observed at weaning along with reduced numbers of females per litter[29]. Both of these reports contrast with the current study in which cryptorchidism carriers were purposely excluded from the breeding stock and there was no effect on the male:female ratio of live pups at birth. However, if stillbirths and early post-natal deaths were included, there was a decrease in male:female ratio, at the expense of males, accompanied by an increase in female mortality and decline in male mortality. Although previous studies of temporal trends in canine stillbirth rates have shown a significant fluctuation with time (1978–2005), gender specific changes have not previously been described[28].

In the current study, although the contemporaneous increase in cryptorchidism and female mortality likely occurs through a separate mechanism, a lethal effect on females induced by genes that cause cryptorchidism has previously been proposed[30]. However, this contention was put forward to account for a 'high sex ratio' in favour of males in litters with cryptorchids, which contrasts with the reduced proportion of males per litter reported in the current study. Notwithstanding, the rise in cryptorchidism, fall in male:female ratio and increased female mortality all alter with time and the former is also related to declining sperm quality for which there is substantial evidence of an environmental trigger.

The dog is probably man's closest companion and by sharing the same habitat, is likely to be exposed to similar environmental conditions including environmental chemicals. This was especially true in the current study since the dogs lived in homes with their handlers. Twelve chemicals detected in adult dog testes (DEHP, 7 PCB congeners, 4 PBDE congeners) were also detected in the 15 commercially available dog foods analysed. Since this indicated a possible food source of exposure, two chemicals (PCB153 and DEHP) were selected for further *in vitro* study on the basis of (1) high abundance in both testis and some pet foods and (2) both chemicals, or their metabolites, are reported to be present in seminal plasma of farm animals and in men at similar concentrations to those reported in the current study[31,32]. In addition, dietary/oral exposure to DEHP or PCB153 alters semen quality in animal models[33,34] and human male infertility has been associated with elevated levels of a mix of PCB congeners in seminal plasma and DEHP[35]. In the current study, 6 PCB congeners and 4 PBDE congeners were detected in entire ejaculate pools at concentrations greater than that observed in the testes. Since testicular concentrations of PCB 153 and DEHP directly impacted on sperm function and viability, it is axiomatic that the higher concentrations found in seminal plasma will similarly alter the parameters measured.

In the current study, we recognise that the lowest chemical concentration used did not equate with a "no adverse effect level (NOAEL)". Although a wider range would be preferable, we have shown effects of PCB153 and DEHP at low environmentally relevant concentrations. The mechanism by which PCB153 and DEHP alter sperm motility and viability is uncertain. Indeed, previous studies of PCB and DEHP effects on sperm quality have yielded mixed results. For example, in the human, a number of studies report an inverse relationship between serum PCBs and sperm quality[36]. In separate studies of seminal ECs across human populations selected on the

basis of variable organochlorine exposure, increased seminal PCB153 was consistently associated with reduced sperm motility[37,38]. In contrast, the sum of 4 PCB congeners in human blood plasma, including PCB153, has been reported to be lower in samples from men with infertility and, although negatively associated with testosterone, was positively associated with sperm quality[39]. Although this latter study is consistent with the positive effect we report of PCB153 on sperm motility, establishing a clear effect of single or multiple PCB congeners is complex, likely due to effects of confounding blood and seminal ECs. Similar mixed results have been observed in relation to DEHP and sperm quality. For example, one study from China reported that human urinary phthalate metabolites, measured as an index of DEHP exposure, were associated with increased sperm DNA damage and apoptosis[40]. In contrast, a separate study from the USA found very little association between DEHP metabolites and a range of sperm parameters including motility[41]. A further study from Sweden however, reported a negative correlation between urinary DEHP metabolites and sperm motility[42]. Phthalate esters have also been measured directly in human semen and reported to be inversely associated with sperm motility[43]. Notably, the authors report that the same inverse relationship was recapitulated *in vitro* when semen concentrations of phthalates were added directly to sperm. These findings support our *in vitro* findings of an inhibitory effect of DEHP on parameters of sperm motility.

In the current study, although the chemical effect on any independent parameter of sperm motility was subtle, the consistent differing effects of PCB153 and DEHP on all four motility parameters may have greater physiological relevance. Both chemicals are known endocrine disruptors with PBDE and PCB considered to have pro-estrogenic or anti-androgenic activity[44] whereas DEHP and its primary metabolite mono-2-ethylhexyl phthalate (MEHP) are reported to exhibit pro- and anti-androgenic activity respectively[45,46]. The differing effects that PCB153 and DEHP have on sperm motility may reflect their respective actions on estrogen or androgen receptors, both of which are expressed by sperm[47,48]. However, it is recognised that many environmental chemicals operate through other endocrine and non-endocrine mechanisms, one or more of which may account for the direct effects on reproductive function. In support of this, neither chemical blunted LH stimulated testosterone secretion however acute direct effects were observed on sperm motility. In the human, environmental concentrations of DEHP and PCB153 have previously been reported to reduce sperm motility[49,50] and interactions have been reported for some PCBs and phthalates mediated by PCB metabolites and enzymes important for phthalate metabolism[51]. With respect to DEHP exposure *in vivo*, the main anti-androgenic effects reported are attributed to its primary metabolite MEHP[52]. For example the exposure of canine testis interstitial cell suspension cultures to MEHP both inhibits hCG induced testosterone secretion and increases basal testosterone secretion[53]. In the current study, although DEHP adversely affected motility, vitality and DNA fragmentation, it had no anti- or pro-androgenic effect on testosterone secretion. This likely reflects the use of DEHP rather than MEHP and/or the lack of metabolic breakdown in culture, as reported for cultured rat fetal testis cells[46] or relatively low levels of activity as reported in human testis extract[54]. The lack of a pro-androgenic response may reflect the use of canine adult testis explants rather than fetal or adult dispersed cells as described above[46,53]. Although we recognise that expanding our study to encompass the effects of PCB and DEHP metabolites on sperm would help consolidate the mechanism, our data are indicative of an interaction between PCB153 and DEHP as reported by others. Furthermore, our observation of an increase in sperm DNA fragmentation and reduced viability, as measured by the hypo-osmotic swelling test, supports previous studies on human sperm showing similar effects and also, increased urinary phthalate concentrations are reported to be associated with lower sperm counts and altered morphology[55,56]. Notwithstanding, it is recognised that the response of sperm to chemicals *in vivo* will inevitably reflect exposure to a more complex mixture of pollutants, many of which may interact. Thus establishing clinical relevance and 'cause and effect' would require the generation of a mixture of chemicals representative of what is present in semen and/or testis.

In conclusion, this study demonstrates that in a population of stud dogs, sperm motility has declined over a 26 year period. Although the mechanism remains to be determined, we have shown that chemicals present in testis and ejaculate directly affect sperm function and viability. Since the increased incidence of cryptorchidism coupled with declining sperm quality in males is indicative of canine "testicular dysgenesis syndrome", the domestic dog may be a useful sentinel for the study of environmental influences on human male fertility.

Methods

Animals and sample collection. All sperm and testis collections and measurements were performed in accordance with relevant guidelines and regulations and all experimental protocols were approved by the University of Nottingham's Animal Welfare and Ethical Review Body. All samples were collected as part of routine breeding management. Sperm was collected as part of routine reproductive examination of stud dogs and residual canine testis was collected from normal healthy dogs (n = 35) during routine castrations and subject to full owner consent. Testis samples were stored at −20 °C until testing.

Monitoring of canine sperm motility, morphology and total sperm output (1998–2014). Sperm was collected annually from stud dogs used in an assistance dog breeding programme. The dogs were of five breed types; Labrador retriever, golden retriever, curly coat retriever, border collie and German shepherd. All dogs lived with their handler and visited the breeding centre for the purpose of breeding or temporary boarding. The number of dogs within the programme varied each year throughout the period of study (n = 42 to 97) and data were available on a total of 1925 ejaculates from 232 different dogs. All semen was collected and evaluated by the last author (GCWE) or one of three technicians trained by him. Collections were performed in the absence of a teaser bitch by digital manipulation of the penis as previously described[57]. The percentage of sperm with normal forward progressive motility (Percentage Normal Motility:PNM), the percentage of morphologically normal live spermatozoa (Percentage Normal Sperm:PNS) and the total sperm output (TSO) was measured using standard

techniques as consistently outlined in the 1987, 1992 and 2010 WHO reference manuals and previously applied to canine semen[58–61]. Morphology slide smears were prepared by mixing a sample of sperm rich ejaculate with nigrosin/eosin at a ratio of 1:4 (ejaculate to stain) for 2 minutes. A histological smear of the mix was examined using a phase contrast microscope at 640 X magnification (oil emersion)[60,62]. Some of the dogs were examined on several consecutive years of the study, commencing when they entered the breeding programme until they left the breeding programme.

In 1998, it was clear that the PNM for the population was significantly lower than it had been in the majority of the 10 preceding years. Therefore in years 1999 to 2001, dogs with poor semen quality (dogs with any of the following measurements: <60% normal forward progressive motility, <60% morphologically normal sperm, <150 million sperm) were removed from the programme. This resulted in an overall improvement in the semen quality of dogs in the programme, especially TSO. On the basis of this intervention, trends in semen quality were evaluated and data are presented as two separate periods from 1988–1998 and 2001–2014 inclusive.

Sperm collection for measurement of and exposure to environmental chemicals. In order to measure chemicals in canine sperm, ejaculate was collected from 14 different stud dogs from the same population in which the decline in semen quality was identified. The sperm rich fraction of each (fraction 2) was collected and two pools of 5 samples and one of 4 samples generated for analysis.

To test the effects of environmental chemicals on parameters of sperm function and viability, ejaculates were collected from the same population of breeding dogs but from a separate cohort of seven normal healthy stud dogs aged between 2 and 8 years. An abstinence period of a minimum of one week was observed and all ejaculates were subjected to the routine fertility assessment previously described: motility, volume (ml), sperm concentration, total sperm output and total number live sperm.

Chemical measurements. Adult dog testes (n = 35), canine ejaculate (n = 14) and a range of commercially available dog foods (dry dog biscuit: n = 13; canned wet meat: n = 12) were tested in an ISO17025 accredited laboratory for PCB congeners (28, 52, 101, 118, 138, 153, 180), PBDE congeners (28, 47, 99, 100, 153, 154, 183) and DEHP. The analytical methods used to determine concentrations used standardised extraction protocols and gas chromatography (GC) analysis as previously described[63,64]. Mean testicular concentrations (MTC) were calculated in a cohort of testes (n = 34: PCB, n = 25: DEHP) as a determination of background tissue concentrations which equate to environmental levels.

Preparation of sperm and chemicals for *in vitro* analyses. PCB153 and DEHP were tested individually or in combination at a final concentration of 2X, 10X and 100X mean testis concentration. Chemicals were dissolved in either 100% ethanol or DMSO and diluted to 0.02% in Phosphate Buffered Saline (PBS) [sperm motility and vitality studies: DEHP 0.2% ethanol][65]. Each sperm sample was diluted (20:1) in PBS and equal volumes of sperm and chemical solutions were mixed and incubated for subsequent analyses. Control sperm cultures for motility, HOS (n = 4) and DNA fragmentation (n = 10) were incubated in PBS with 0.1% solvent only (working concentration).

DNA fragmentation (sperm chromatin dispersion assay). Diluted semen samples were cultured for 0, 2 and 4 hours with concentrations of DEHP or PCB-153 equivalent to 2x, 10x and 100x those measured in testes. Each semen sample was individually combined with an equal volume of 1% low-melting point agarose. A 15 μl aliquot of the sperm-agarose suspension was pipetted onto a polysine slide (Thermo Fisher Scientific, Loughborough, UK), pre-coated with a 1% agarose solution, and after application of a coverslip, it was left to solidify at 4 °C for 5 minutes. Following coverslip removal, slides were immersed in acid denaturing solution (0.08 M HCl) for 7 minutes. Slides were then incubated with lysing reagent (0.8 M DTT, 0.4 M Tris, 2 M NaCl, 1% triton-X) for 20 minutes. Lysing of cells was stopped by immersion in dH2O for 5 minutes prior to a series of sequential ethanol dehydration steps (70%, 95% and 100%). Visualisation occurred via submersion of slides in Diff-Quik (Eosin-Y) followed by Methylene Blue for 7 minutes and analysed via brightfield microscopy at 640X magnification (oil emersion). A minimum of 200 sperm were counted and percent fragmentation calculated. Sperm not showing the characteristic halo, indicative of chromatin dispersal, were considered fragmented.

Hypo-osmotic swelling test (HOST). As previously described[66], a hypotonic solution (150 mOsm/l1^{-1}) was prepared using fructose (Sigma, Poole, UK), sodium citrate (Sigma, UK) and reverse osmosis water. The sperm rich portion of the ejaculate (0.1 ml) was incubated in 1.0 ml of hypo osmotic solution for 30 minutes at 37 °C. After mixing, 10 ul was smeared onto a glass slide and air dried. Two hundred sperm were observed with a phase contrast microscope at 400X magnification. The assay was carried out on all samples at 0 and 4 hours and the percentage of cells showing osmotic stress calculated.

Computer Assisted Sperm Analysis (CASA). Semen (sperm rich fraction), mixed with chemical culture media, was incubated in a water bath at 37 °C. Ten μl of mixed semen was placed on a Helber counting chamber slide (depth 0.02 mm: Hawksley, UK), covered with a 22 × 22 mm coverslip and assessed for motility using a Hobson Sperm Tracker (Hobson Tracking Systems Ltd, Sheffield, UK). Standard settings recommended by the supplier were used and each treatment tracked for one minute at four time points (0, 1, 2, 4 hours) as previously described[67]. All CASA parameters were recorded for each chemical (PCB 153, DEHP and mix PCB153/DEHP) at each concentration (x2, x10, x100 mean testis concentration).

Fourteen parameters of sperm motility were measured using the CASA system. These were progressive motility (WHO laboratory manual)[60], straight line velocity (VSL), curvilinear velocity (VCL), average path velocity (VAP),

straightness of track (STR), linearity of track (LIN), amplitude of lateral head displacement (ALH), beat cross frequency (BCF), dance mean (DMN), pause variation of VSL (PFT), Mean angular displacement (MAP), percent active, percent hyperactive and percent motile[60,68,69].

Canine adult testis explant culture and testosterone ELISA. Left and right testes were collected from routine castrations and immediately transferred into pre-warmed serum free Dulbeco's Modified Eagle Medium (DMEM: Sigma-Aldridge Company Ltd) supplemented with high fructose, penstrep (100 ug/ml) and gentamycin (50 ug/ml). After removal of the tunica vaginalis and epididymis, the testis was bisected and a 2.5–3 mm disk (cross section) taken from the centre. A 3 mm biopsy punch was used to generate small explants (<0.5 mm^2) of testis parenchyma from the disk periphery thus avoiding the central rete testis. Fragments of testis were transferred to a 12 well culture plate (3 pieces per well) containing DMEM (100 ul/well) supplemented with high fructose, 10% fetal bovine serum, L-glutamine (200 mM), penstrep (20 ug/ml) and gentamycin (20 ug/ml). Testis fragments were washed (Hera Cell 150i, Thermo Scientific) for 30 minutes at 37 C in 5% CO2 with gentle shaking (500 rpm–Sea Star Digital Shaker: Thomas Scientific, Swedesboro, NJ, USA) to aid diffusion of waste gases. Initially testes were incubated for 6, 12, 18 or 24 hours and processed into Bouins for histological analysis. This enabled the optimal incubation to be determined without excessive tissue necrosis. To establish optimum treatments for testosterone secretion, explants were cultured for 24 h with LH (Fisher Scientific) at 0.1, 1 and 10 ng/ml.

Testis explants from 6 dogs were washed for 30 minutes then cultured for 24 h with 0.1, 1 or10 ng/ml LH both with and without PCB153 or DEHP at 10x or 100x mean testicular concentrations. Positive control explants were incubated with dbcAMP and chemicals were made up in 0.01% DMSO (working concentration). Negative controls were incubated in the absence of LH or chemical but in the presence of 0.01% DMSO. At the end of each culture period, media was collected for testosterone analysis using the Enzo Life Sciences testosterone ELISA kit (Enzo Life Sciences, Exeter, UK); detection limit of 38.3 pg/ml; intra- and interassay coefficients of variation of 17% and 20% respectively.

Statistical analysis. A statistical model was constructed to evaluate the change in semen quality over time whilst accounting for repeated semen measurements within dogs. Conventional multilevel models were specified that accounted for repeated correlation measures[70] using MLwiN version 2.22[71] with semen quality characteristics either modeled directly as a normal response variable or, when appropriate, log transformed, to ensure model residuals followed an approximately normal distribution. Possible confounding effects of the age of the dog on semen quality were tested as linear and polynomial terms. In addition confounding effects of breed (categorical variable), bodyweight (tested as linear and polynomial terms) and sire of dog (included as a random effect) were investigated. Since its inclusion had no substantive effect in any model, sire was not included as a random effect in the final models. Model specification was:

$$\text{Semen Characteristic}_{ij} = \alpha + \beta_{1-n}\text{Year}_{1-n} + \beta_2 X_{ij} + \beta_3 X_j + u_j + e_{ij}$$

where the subscripts i, and j denote the i^{th} repeated measurement of semen quality (Percentage Normal Motility, Percentage Normal Sperm or Total Sperm Output), in the j^{th} dog, α is the regression intercept, Year_{1-n} are dummy variables representing each year of the 'n' study years, with β_{1-n} the related coefficients, X_{ij} are covariates associated with each semen quality reading (such as age of dog at sampling) with β_2 the related coefficients, X_j are covariates associated with each dog (such as breed) with β_3 the related coefficients, u_j is a random effect to account for repeated correlated measures within dogs (assumed to follow a Normal distribution with a mean of zero and variance Ω_u) and e_{ij} is an error term reflecting residual variation between semen quality measurements (assumed to follow a Normal distribution with a mean of zero and variance Ω_e).

Model building followed an exploratory stepwise procedure and explanatory covariates remained in the model when the significance probability was <0.05. Interaction terms between covariates were tested in the model and also remained when $P < 0.05$. Model fit was visually assessed using graphs of residuals at each hierarchical level[70]. The data were examined for points or higher level units with a large influence on parameter values and the models were re-run when necessary, with these points omitted, to examine their effect on parameter values.

Temporal changes in cryptorchidism, male:female ratio and mortality of male and female fetuses were assessed by linear regression analysis using GraphPad prism statistical analysis software. Chemicals (PCB153, DEHP) were tested singly and in combination as a 2 chemical by 3 concentration by 4 times split plot factorial design. Raw data collected from the Hobsons Sperm Tracker was used and comparisons made using a general ANOVA model (Genstat). Data was fitted to the model so that interactions between the concentration of each chemical and time could be assessed. Dogs were considered to be replicate blocks and sperm samples within dogs as the main plots. The main effect of concentration group was tested against the main-plot between-sample error; all other factors and their interactions were tested against the residual sub-plot error.

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Author Contributions

R.G.L. and G.C.W.E. conceived of and designed the study. G.C.W.E. and S.L.F. managed the collection and monitoring of canine semen quality. R.G.L., A.S.B. and S.M.R. managed the collection of testes for *in vitro* studies. A.S.B., R.N.S. and H.M.R. carried out the computer assisted sperm analysis. R.N.S. carried out the chromatin dispersion assay and A.S.B. developed the testis explant culture system. Z.Z. carried out all chemical analyses. R.M. managed data extraction from the electronic database and contributed to the analysis. M.G. and J.C. provided expert statistical advice and contributed to the analysis. R.G.L. wrote the paper and the work was funded by the Guide Dogs for the Blind Association.

Additional Information

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Endocrine disrupting chemicals in dog testis and a range of dog foods: is the dog a sentinel for human exposure?

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Introduction

- Human and canine testicular cancer has increased in the last 40 years[1,2].
- In humans, this has been associated with a decline in sperm quality[3] and reproductive health collectively termed testicular dysgenesis syndrome (TDS)[4].
- Exposure to endocrine disrupting chemicals (EDCs) via diet is implicated in human TDS .

Hypothesis:
EDCs perturb canine reproductive health and fertility.
Aims:
1. Determine levels of EDCs in dog diet and tissue.
2. Assess value of the dog as a model for human reproductive health

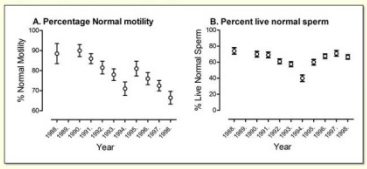
Methods

- Between 1988 and 2010, ejaculates (n=1925) were collected from stud dogs (n=232) at the National Breeding Centre. Two periods of monitoring are presented (1998-1998, 2005-2010).
- Sperm quality parameters (motility, % live normal and output) were assessed by standard techniques.
- Polychlorinated Biphenyls (PCB: 7 congeners), Polybrominated Diphenylethers (PBDE: 7 congeners) and Di(2-ethylhexyl) phthalate (DEHP) were measured in commercially available dry (n=14) and wet (n=14) dog foods.
- Canine testicular tissue (n=5) and prostatic fluid (n=3), collected during routine castrations or breeding examinations, were tested for the same EDCs.

Results

Declining sperm quality in dogs

- 1988 to 1998: significant decline in sperm motility and % live normal sperm (P<0.05: Fig.1).



Multilevel statistical modelling revealed no effect of age, breed, body weight or sire

Figure 1: Sperm motility and % live normal sperm 1988 - 1998

- 2005 to 2010: significant decline in sperm motility & total output (Fig.2).

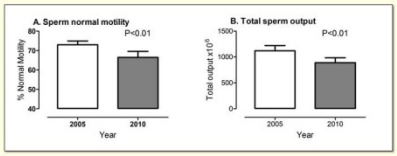


Figure 2: Sperm motility and total sperm output 2005 v 2010

EDC levels in testis and prostatic fluid

- **Testis:** all PCB congeners detected with 3 congeners at relatively higher levels in all samples (PCB 28: 5/5, PCB 101: 4/5, PCB 153 4/5) (Fig. 3A).
- PBDE 47 was detected in testis at higher levels than the other congeners (Fig. 3B).
- DEHP was present in all 5 testis samples evaluated (Fig. 3C).
- **Prostatic fluid:** Concentrations of all EDCs were below detectable limits.

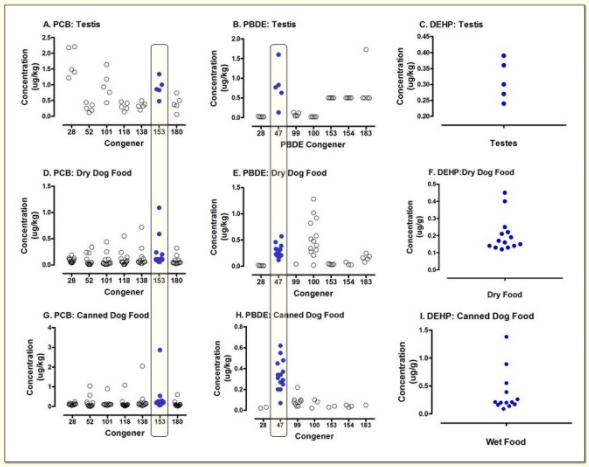


Figure 3: PCB, PBDE congeners and DEHP in testis and dog food. Each circle represents a single testis or food sample. Blue circles: chemicals with relatively high levels in testis and food.

EDC levels in a range of commercial dog foods

- **Dry and canned food PCB:** All congeners detected (Fig. 3D,G). Specific brands had higher levels of PCB congeners 153, 138, 118, 101, and 52 (Fig. 3D,G).
- **Dry and canned food PBDE:** congener 47 was relatively high (Fig. 3E,H) PBDE 100 was higher in dry food only (Fig. 3E).
- **DEHP:** present in all samples and elevated in 3 brands of canned food (Fig. 3C,F,I).

Chemical profile in testis and food

- PCB153, PBDE 47, DEHP found at relatively high levels in testis, dry and canned dog food (Fig. 3).

Conclusion

- Canine sperm quality is declining in parallel with that seen in humans.
- EDCs are present in dog testis and food.
- For some EDCs, high levels in feed ARE reflected in testis levels; for others they ARE NOT.
  - Relatively high levels of DEHP, PBDE 47 and PCB 153 were found in testis and food.
  - Different congener profiles are indicative of non dietary sources of EDCs.
- The dog may be a sentinel for human exposure to EDCs.
- The dog may be a model for the study of human reproductive health.

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Acknowledgement: Funding provided by Guide Dogs for the Blind Association, Nottingham Trent University, University of Nottingham

Changes in canine sperm function after exposure to testicular concentrations of PCB153 and DEHP

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Introduction

- Human testicular dysgenesis syndrome, including declining sperm counts, is associated with exposure to endocrine disrupting chemicals (EDCs).
- Comparable temporal trends to those seen in humans have been reported in the dog.
- Diet has been identified as one route of EDC contamination.
- We have observed a temporal decline in canine sperm quality.

Hypothesis:
Declining canine sperm quality is associated with dietary exposure to EDCs.
Aims:
1. Determine levels of specific EDCs in testicular tissue and canine diet.
2. Determine the effects of environmental concentrations of EDCs on canine sperm viability and motility.

Methods

- **Chemical screening:**
  - Canine testicular tissue (n=5) screened for Polychlorinated Biphenyls (PCB: 7 congeners), and Di(2-ethylhexyl) phthalate (DEHP).
  - Dry (n=14) and wet (n=14) dog foods screened as above.
  - PCB153 and DEHP, detected in canine testis and food, selected for analysis.
- **Animals:**
  - Testes collected from routine castrations
  - Canine sperm collected dogs of proven fertility (n = 7).
- **Treatments**:
  - Balanced 2 chemical by 3 concentration by 4 times split plot factorial design.
  - Fresh sperm exposed to PCB153 and DEHP singly and in combination
  - Concentrations = 0, 2x, 10x, 100x mean testis levels. Time = 0, 30, 60, 240 mins
- **Experiments:**
  - The hypo osmotic swelling test (HOS) measured at 4 time points
  - Motility: computer assisted sperm analysis (Hobson's Tracking Systems Ltd., UK).

Results

EDC Levels in testis and a range of commercial dog foods

- PCB: all congeners detected [Fig 1A: testis, 1B,C: food].
- DEHP: detected in all samples [Fig 1D: testis, 1E,F: food].

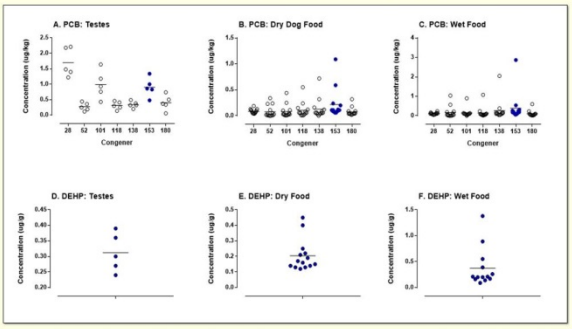


Figure 1: EDC detecting in testes and commercial dog food. PCB (7 congeners) and DEHP levels in (A) testis (n=5), (B) dry dog food (n=14) and (C) wet dog food (n=14). DEHP levels in (D) testis (n=5), (E) Dry food(n=14) and (F) Wet food(n=14) . Each circle represents a single testis or food sample. Blue circles: chemicals with relatively high levels in testis and food. Bar indicates mean value.

Selected chemicals for sperm analysis

- PCB153 and DEHP consistently detected in testis and food [Figure 1].
- Testis (n=5; mean conc. ± SEM); sperm viability and motility experiments
  - Testis: PCB153: 0.9 ± 0.14 µg/kg; DEHP: 0.31 ± 0.03 µg/g,
  - Dry Dog Food: PCB153: 0.22 ± 0.08 µg/kg; DEHP: 0.20 ± 0.03 µg/g),
  - Wet Dog Food: PCB153: 0.39 ± 0.19 µg/kg; DEHP: 0.37 ± 0.10

Sperm Viability

PCB153:

- Low concentrations reduce percentage of HOS positive sperm (PCB x conc: P=0.026) [Fig. 2A]

DEHP:

- Reduced the number of HOS positive sperm at 240 minutes (DEHP x time: P= 0.041) [Fig. 2B]

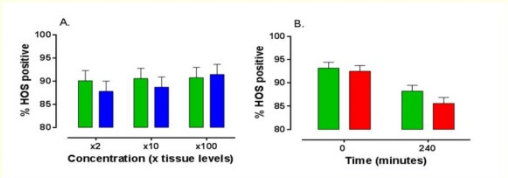


Figure 2 : Effects of PCB 153 and DEHP on HOS of canine sperm . (A) PCB 153 at 2, 10, 100 x mean testes concentration, (B) DEHP at time points 0 and 240 minutes. Dogs: n=7; values = mean (+SED).

Sperm Motility

Percent motile

- PCB153 ↑(P=0.031) [Fig 3A]

Percent linearity

- PCB153 ↑(P=0.036), DEHP ↓ (P=0.031)
- Suggestion that effects might vary with concentration and presence of other chemical (P=0.059) [Fig 3B]

Straight line velocity

- PCB153 ↑(P=0.051), DEHP ↓ (P=0.037)
- Effect varies with concentration and presence of other chemical (P=0.046) [Fig 3C]

Percent straightness

- DEHP ↓ (P=0.032) [Fig 3D]

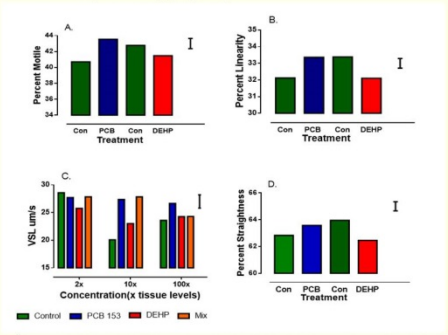


Figure 3: Effects of PCB 153 and DEHP individually and combined (mix) on sperm motility. (A) percent motile sperm, (B) percent linearity, (C) straight line velocity (VSL) , (D) percent straightness. Sperm were exposed to one of three chemical concentrations (2, 10, 100 x mean testes concentration). Significant chemical and/or chemical x concentration effects are shown. Dogs: n=7; values = mean (± SED).

Conclusion

- PCB congeners and DEHP are detectable in canine testes and diet.
  - PCB 153 and DEHP are detectable in both.
- Testicular concentrations of PCB 153 and DEHP perturb sperm membrane integrity and motility.
- Dietary EDCs may be associated with declining canine fertility.

Acknowledgements: Funding provided by Guide Dogs for the Blind Association, Nottingham Trent University, University of Nottingham

Environmental concentrations of chemical pollutants alter sperm vitality, motility and morphology: comparative studies in the human and dog.

The University of Nottingham
UNITED KINGDOM · CHINA · MALAYSIA

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Introduction

Human testicular dysgenesis syndrome, including declining sperm count, is associated with exposure to environmental chemical pollutants many of which are endocrine disrupting chemicals (EDCs).

Comparable adverse temporal trends in reproductive health and fertility have been reported in the dog.

Environmental chemical pollutants have been detected in canine testis.

Hypothesis:

Environmentally relevant concentrations (testicular) of chemical pollutants will perturb sperm quality and function in both dogs and humans.

Aims:

To investigate the effects, *in vitro,* of two chemical pollutants on canine and human sperm motility, morphology and vitality.

Methods

Chemical pollutants:

- Polychlorinated Biphenyl 153 (PCB-153) and Di-ethyl-hexyl Phthalate (DEHP) selected for culturing with canine and human spermatozoa.

Canine and human sperm samples:

- Canine sperm rich fraction collected from dogs of proven fertility (n = 7)
- Human ejaculates (n=9) from 4 anonymous donors with proven fertility.

Experimental measurements, treatments and statistical analysis:

- **Parameters:** Motility, Vitality and Morphology (≥ 200 sperm analysed).
- **Chemical treatment**: PCB153 and DEHP singly and in combination (mix) or control (no chemical) applied to culture tubes from each sperm sample.
- **Concentrations**: 2, 10, 100x mean canine testicular levels (MTL) applied to different samples:
  - *[PCB 153: 0.9 ± 0.14 µg/kg(n = 8); DEHP: 0.31 ± 0.03 µg/g (n=5)]*
- **Sampling time**: Dog (0, 30, 60, 240), Human (0, 240) minutes.
- **Design**: 4 chemical treatment by 3 concentration by 4 time points (canine) 2 time points (human).
- **Analysis**: Genstat generalised linear mixed model: normal or binomial errors;
  - Fixed effects: *treatment x concentration x time*
  - Random effects: *sample and treatment tube within sample*

Results

Morphology – Human: (VitalStain™)

- Proportions of morphologically normal & abnormal sperm decreased and increased respectively (P=0.007) [A]. This was primarily due to PCB 153.
- Proportion of total sperm showing head abnormalities significantly increased by treatment (P=0.01) [B]. This was primarily due to DEHP.

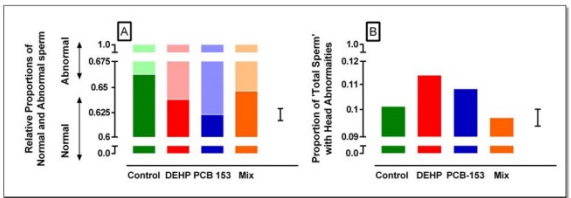


Figure 1: Morphology of human sperm following culture with DEHP, PCB 153 or a mix of both chemicals. [A] Morphologically 'Normal & Abnormal' sperm (proportion of total). [B] Sperm with 'Head Abnormalities'. Total sperm = live + dead. Values are predicted mean proportions. Error bar = 1 SED.

Morphology - Canine:

- No effect observed.

Motility – Human:

(Computer Assisted Sperm Analysis (CASA) - Sperminator®)

- Treatment effect on %age of sperm with Category B motility (P<0.001) [B].
- No change observed in Categories A, C or D sperm motility [A, C, D].

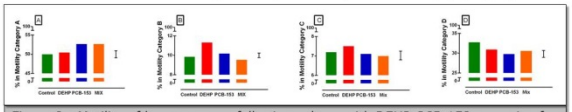


Figure 2 : Motility of human sperm following culture with DEHP, PCB 153 or a mix of both. [A] Category A (>25 µM/second); [B] Category B (5-25 µM/second); [C] Category C (<5 µM/second): [D] Category D (static). Error bar = 1 SED.

Motility – Canine: (CASA - Hobson Tracker)

- PCB 153 increased and DEHP decreased a range of motility parameters.
- Significant treatment effects observed for % linearity, % motility (PCB only), % straightness (DEHP only) and straight line velocity (Figure 3).

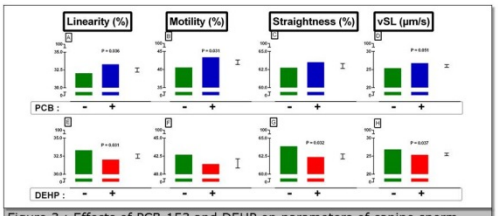


Figure 3 : Effects of PCB 153 and DEHP on parameters of canine sperm motility.
Linearity, motility, straightness and straight line velocity (vSL) effects shown for PCB [A-D] and DEHP [E-H]. Error bar = 1 SED.

Vitality – Human: (VitalStain™)

- No effect on relative proportion of live and dead sperm [A].
- Proportions of live normal sperm ↓ and dead abnormal sperm ↑ by treatment (P=0.007, 0.006 respectively) [B,C].

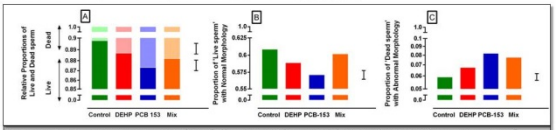


Figure 4: Human sperm vitality following culture with DEHP, PCB 153 or a mix of both. [A] 'Total Live & Dead' sperm, [B] 'Live Normal' sperm [C] 'Dead Abnormal' sperm. Values are predicted mean proportions. Error bar = 1 SED

Vitality – Canine: (Hypo-osmotic swelling test (HOS))

- Sperm vitality significantly reduced by PCB153 (Treatment x concentration: P=0.049) [A] and DEHP (P=0.002) [B].

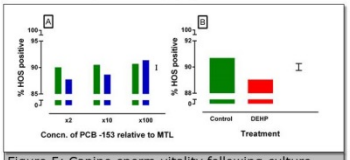


Figure 5: Canine sperm vitality following culture with DEHP & PCB 153. [A] PCB 153 at 2, 10, 100 x Mean Testis Level, [B] DEHP. Values are mean %. Error bar = 1 SED

Conclusion

- Exposure of spermatozoa to environmentally relevant concentrations of chemical pollutants effects sperm vitality and motility in the dog and sperm morphology and motility in the human.
- Although dogs are sentinels of human exposure, they appear to respond differently to the same chemical insult (e.g. motility parameters).
- The underlying mechanism for this species difference is unknown.
- These effects may be associated with adverse temporal trends reported in human and canine male sperm quality.

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• Ethical approval: Ethics committees from Nottingham Medical School and School of Veterinary Medicine and Science.

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Characterisation of the dog as a sentinel species for human exposure to endocrine disrupting chemicals

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Introduction

Human: Exposure to environmental chemicals (ECs) has been associated with a decline in sperm counts and an increased incidence of testicular cancer. Since these problems occur together in specific geographical areas (national & international) they are thought to have a common aetiology.

Dog: A population of stud dogs at the GDBA have shown a decline in sperm quality that parallels that reported in the human.

Hypothesis: Declining canine sperm quality and reproductive health is associated with exposure to environmental chemicals (ECs).

Aims: to determine

- Testicular concentrations of ECs in canine testis.
- Effects of testicular chemicals on sperm DNA quality.
- Geographical variations in testicular EC profiles and Sertoli cell numbers

Methods

Chemical screening:

- Polychlorinated biphenyls (PCB: 7 congeners)
- Di(2-ethylhexyl) phthalate (DEPH)
- UK Canine adult testes (routine castrations: n=35) analysed from:
  - West Midlands (WM: n=12)
  - East Midlands (EM: n=9)
  - SE England (SE: n=14)
- Scandinavia canine adult testes:
  - Denmark (n=10)
  - Finland (n=10)
- Canine food:
  - Dry (n=14), Wet (n=14)
- PCB153 and DEHP, detected in testis and food, selected for analysis

Sperm culture experiments:

- Canine sperm collected from dogs of proven fertility (n = 10)
- Chemicals applied at 2, 10, 100x mean testicular levels (MTL)
- Treatments: PCB153 and DEHP singly or control (no chemical)
- Parameters: Motility, Vitality & DNA integrity (≥ 200 sperm analysed)
- Sampling time: 0, 120 and 240 minutes
- Design: 3 chemical treatment by 3 concentration by 3 time points
- Analysis: ANOVA (orthogonal analysis; Genstat)

Testicular Sertoli cell analyses:

- UK: n=20 [WM (5), SE (5) & EM (10 from 2 regions)]
- Scandinavia: n=10 [Denmark (5), Finland (5)]
- SC parameters: counts (tubule area) & % tubule cellular area stained

Results

EC Levels in testis and a range of commercial dog foods

- PCB: all congeners detected [Fig 1A: testis, 1B,C: food].
  PCB153 mean testis level = 0.063 ± 0.01 µg/kg
- DEHP: detected in all samples [Fig 1D: testis, 1E,F: food].
  DEHP mean testis level =0.182 ± 0.044 µg/g

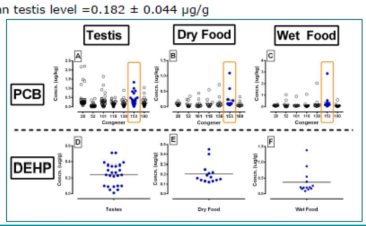


Figure 1 : ECs detected in testes and commercial dog food. PCB (7 congeners) and DEHP levels in (A) testis (n=35 & 25 respectively), (B) dry food (n=14) and (C) wet food (n=14). Each circle represents single testis or food sample. Blue circles: chemicals consistently detected in testis and food.

Motility (% total): (Manually assessed)

- Significant ↓ with treatment (P<0.001) [Fig 2A]
- Suggestion of ↓ with individual chemicals (P=0.085) [Fig 2B]

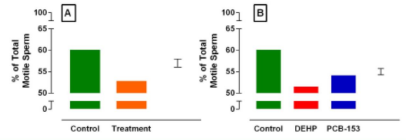


Figure 2 : Effects of PCB 153 and DEHP on canine sperm percent motility. Error bar = 1 SED.

Vitality: (Nigrosin:Eosin Stain)

- Significant ↓ with individual chemicals (P<0.01) [Fig 3]

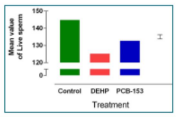


Figure 3: Canine sperm vitality following culture with DEHP & PCB 153. Error bar = 1 SED

DNA Integrity: (Sperm Chromatin Dispersion Assay)

- Fragmentation ↑ with individual chemicals (P=0.055) [Fig 4]

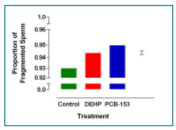


Figure 4: Effects of PCB-153 and DEHP on sperm DNA Integrity. Values = predicted mean proportions. Error bar = 1 SED.

Testicular ECs & Sertoli cell analyses:

- UK testes:
  - ΣPCB congeners: WM > SE & EM [Fig 5A: P<0.05]
  - SC numbers: WM < SE & EM [Fig 5B: P<0.01]
- Scandinavian testes:
  - DEHP: Denmark > Finland [Fig 5D: P<0.05]
  - SC % area stained: Denmark > Finland [Fig 5F: P<0.01]

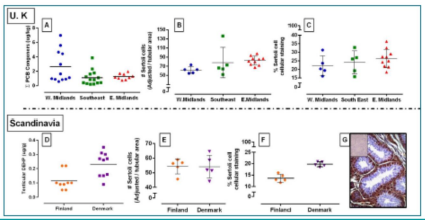


Figure 5: Regional variation in testis EC concentrations and Sertoli cell parameters: UK (A,B,C) & Scandinavia (D,E,F). Chemicals: ΣPCB (A) & DEHP (D), SC numbers adjusted per tubule (B,E) & Percent SC cellular staining (C,F) by vimentin immunohistochemistry (G). Error bar = 1 SED.

Conclusion

- Testicular concentrations of ECs perturb sperm quality and exhibit regional variation in profile with altered testicular morphology.
- These data support the hypothesis that declining canine fertility has an environmental aetiology.